EXHIBIT 1

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Applicants: Silviu Itescu
Serial No.: 10/693,480
Filed: October 23, 2003

(54) Title: REGENERATION OF ENDOGENOUS MYOCARDIAL TISSUE BY INDUCTION OF NEOVASCULARIZATION

(57) Abstract: This invention provides a method of treating a disorder of a subject's heart involving loss of cardiomyocytes which comprises administering to the subject an amount of an agent effective to cause cardiomyocyte proliferation within the subject's heart so as to thereby treat the disorder. This invention further provides the instant method wherein the agent is human endothelial progenitor cells, G-CSF, GM-CSF, SDF-1, and IL-8. This invention also provides methods of determining the susceptibility of a cardiomyocyte in a subject to apoptosis.

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REGENERATION OF ENDOGENOUS MYOCARDIAL TISSUE BY INDUCTION OF NEOVASCULARIZATION

This application claims priority of U.S. Serial No. 10/128,738, filed April 23, 2002, the contents of which are hereby incorporated by reference.

Throughout this application, various publications are referenced in parentheses by arabic numbers. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Background

Healing of a myocardial infarct is complicated by the need for viable myocytes at the peri-infarct rim to undergo compensatory hypertrophy in order to increase pump function in response to the loss of infarcted tissue (1,2). This initiates a process termed cardiac remodelling which is characterized by apoptotic loss of hypertrophied myocytes, expansion of the initial infarct area, progressive collagen replacement, and heart failure (3-6). We have recently put forward the hypothesis that hypertrophied cardiac myocytes undergo apoptosis because the endogenous capillary network cannot provide the compensatory increase in perfusion required for cell survival (7).

Vascular network formation is the end result of a complex process that begins in the pre-natal period with induction

of vasculogenesis by hemangioblasts - cells derived from the human ventral aorta which give rise to both endothelial and hematopoietic elements (8-11). Cells which can differentiate into endothelial elements also exist in adult bone marrow (12-14) and can induce vasculogenesis in ischemic tissues (15-17). In the adult, new blood vessel formation can occur either through angiogenesis from pre-existing mature endothelium or vasculogenesis mediated by bone marrow-derived endothelial precursors. Recently, we identified a specific population of endothelial progenitor cells (angioblasts) derived from human adult bone marrow which has phenotypic and functional characteristics of embryonic angioblasts (7). We showed that intravenous administration of these cells resulted in selective homing to ischemic myocardium, induction of infarct bed vasculogenesis, prevention of peri-infarct myocyte apoptosis, and significant improvement in myocardial function (7).

We recently discovered that CXC chemokines containing the ELR motif regulate migration of human bone marrow-derived endothelial progenitor cells to sites of tissue ischemia. Moreover, since selective bone marrow homing and engraftment of hematopoietic progenitors depends on CXCR4 binding to SDF-1 expressed constitutively in the bone marrow (28-30), we demonstrated that interruption of CXCR4/SDF-1 interactions could redirect trafficking of human bone marrow-derived endothelial progenitor cells to sites of tissue ischemia, thereby augmenting therapeutic vasculogenesis. Our results indicated that CXC chemokines, including IL-8, Gro-alpha, and SDF-1, play a central role in regulating human adult bone marrow-dependent vasculogenesis.

Recent observations have suggested that a second

compensatory response of viable cardiomyocytes is to proliferate and regenerate following injury (18,19). We have previously shown that pro-angiogenic fatcors, such as endothelial progenitor cells at a minimum concentration can

5   induce vasculogenesis. Here we disclose the surprising result that careful dosing of endothelial progenitor cells/other pro-angiogenic agents can induce cardiomyocyte proliferation also, and this proliferation can be enhanced by manipulating CXCR4/stromal derived factor-1

10   interactions. Moreover, we show that the mechanisms for this enhanced proliferation is via modulation of mRNA expression of a number of stress-inducible genes involved in cardiomyocyte apoptosis and cell-cycling.

Summary

This invention provides a method of treating a disorder of a subject's heart involving loss of cardiomyocytes which
5   comprises administering to the subject an amount of an agent effective to cause cardiomyocyte proliferation within the subject's heart so as to thereby treat the disorder.

This invention further provides the instant method wherein
10   the agent is human endothelial progenitor cells.

This invention further provides the instant method comprising administering an effective amount of a second agent that increases the cardiomyocyte proliferation caused
15   by the human endothelial progenitor cells.

This invention also provides a method of determining the susceptibility of a cardiomyocyte in a subject to apoptosis comprising:
20   (a)   quantitating the expression a peroxiredoxin in the cardiomyocyte;
          (b)   quantitating the expression of Vitamin D3 Up-Regulated Protein-1 in the cardiomyocyte; and
          (c)   determining the ratio of peroxiredoxin
25   expression: Vitamin D3 Up-Regulated Protein-1 expression, wherein a low ratio indicates a high susceptibility of the cardiomyocyte to apoptosis and a high ratio indicates a low susceptibility of the cardiomyocyte to apoptosis in the subject.

BRIEF DESCRIPTION OF THE FIGURES

<u>Figures 1A-1D</u>: IL-8/Gro-Alpha CXC Chemokines Regulate Migration Of Human Endothelial progenitor cells (angioblasts) To Myocardial Tissue In Vivo And Subsequent Development Of Vasculogenesis.

(A) DiI-labelled. human endothelial progenitor cells (angioblasts)(>98% CD34+ purity) injected intravenously into nude rats infiltrate rat myocardium after coronary artery ligation and infarction but not after sham operation at 48 hours.

(B) Migration of human endothelial progenitor cells (angioblasts) to ischemic rat myocardium is inhibited by mAbs against either rat IL-8 or the IL-8/Gro-alpha chemokine family receptors CXCR1 and CXCR2 (all $p < 0.01$), but not against VEGF or its receptor Flk-1 (results are expressed as mean + sem of three separate experiments).

(C) Masson's trichrome stain of rat myocardial infarct bed at two weeks after LAD ligation demonstrating diffuse increase in matrix deposition and few capillaries in representative animal injected with saline (x400), diffuse increase in capillaries (arrowheads) and reduction in matrix deposition in representative animal injected with human bone marrow-derived endothelial progenitor cells (x400), and reduction in capillary numbers in representative animal injected with human endothelial progenitor cells (angioblasts) together with mAb against human CXCR1/2 (x400).

(D) Intracardiac injection of IL-8 or SDF-1 at 1μg/ml significantly increases in vivo chemotaxis of DiI-labelled human endothelial progenitor cells (angioblasts)(98% CD34+ purity) into non-ischemic rat heart in comparison with injection of saline or stem cell factor (SCF), $p < 0.01$ (results are expressed as mean + sem of three separate experiments). Below is shown representative fluorescence

microscopy of intravenously-injected DiI-labelled human endothelial progenitor cells infiltrating non-ischemic rat heart after intracardiac injection with saline, IL-8 or SDF-1.

Figures 2A-2C: Blocking CXCR4/SDF-1 Interactions Redirects Intravenously Injected Human Endothelial progenitor cells From Bone Marrow To Ischemic Myocardium.

(A) the proportion of human CD34+CD117^{bright} endothelial progenitor cells (angioblasts) in rat bone marrow 2-14 days after intravenous injection is significantly increased following ischemia induced by LAD ligation (results are expressed as mean + sem of bone marrow studies in three animals at each time point).

(B) and (C) depict the effects of mAbs against CXCR4, SDF-1 or CD34 on trafficking of human CD34+ endothelial progenitor cells (angioblasts) to rat bone marrow and myocardium following LAD ligation. Co-administration of anti-CXCR4 or anti-SDF-1 significantly reduced trafficking of intravenously injected human CD34+ cells to rat bone marrow at 48 hours and increased trafficking to ischemic myocardium, whereas anti-CD34 mAb had no effect (results are expressed as mean + sem of bone marrow and cardiac studies performed in three LAD-ligated animals at 48 hours after injection).

Figures 3A-3F. Redirected Trafficking Of Human Endothelial progenitor cells (angioblasts) To The Site Of Infarction Induces Vasculogenesis And Protects Cardiomyocytes Against Apoptosis.

(A) Myocardial infarct bed two weeks post-LAD ligation from representative animals in each group stained with Masson's trichrome (upper panel) or immunoperoxidase after binding of anti-CD31 mAb (lower panel). The infarct zones of rats receiving either 10^3 or 10^5 endothelial progenitor cells

(angioblasts) show myocardial scars composed of paucicellular, dense fibrous tissue stained blue by trichrome (x400). In contrast, the infarct zones of rats injected with 10^5 endothelial progenitor cells plus anti-CXCR4 mAb show significant increase in cellularity of granulation tissue, minimal matrix deposition and fibrosis, and numerous medium-sized capillaries of human origin. The infarct zones of rats injected with 2×10^5 endothelial progenitor cells show a similar reduction in fibrous tissue and increase in medium-sized capillaries, and an additional increase in large-sized vessels of human origin.

(B) and (C) show the relationship between the number of human CD117^{bright} endothelial progenitor cells injected intravenously (10^3, 10^5, 10^5 plus anti-CXCR4 mAb, and 2×10^5) and development of rat infarct bed vasculogenesis at two weeks, defined as the mean number of capillaries/high power field (hpf) with medium- or large-sized lumen diameter (respectively, 0.02 mm mean diameter with 3-6 contiguous endothelial lining cells and 0.05 mm mean diameter with >6 contiguous endothelial lining cells). Results are expressed as the mean + sem of at least 15 hpf in three separate experiments.

(B) the groups receiving either 2×10^5 endothelial progenitor cells (angioblasts) or 10^5 endothelial progenitor cells plus anti-CXCR4 mAb demonstrated 1.7-fold higher numbers of medium-sized capillaries compared with the other two groups ($p<0.01$).

(C) the group receiving 2×10^5 endothelial progenitor cells (angioblasts) additionally demonstrated 3.3-fold higher numbers of large-lumen capillaries compared with the groups receiving 10^3 or 10^5 endothelial progenitor cells ($p<0.01$), and 2-fold higher numbers of large-lumen capillaries compared with the group receiving 10^5 endothelial progenitor cells plus anti-CXCR4 mAb ($p<0.01$).

(D) shows that co-administration of anti-CXCR4 mAb together

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with the highest concentration of endothelial progenitor cells (angioblasts), 2×10^5, resulted in a further 23% increase in growth of large-lumen capillaries. More strikingly, there was a further 2-fold increase in

5   capillary numbers when 2×10^5 endothelial progenitor cells were injected intravenously after direct intracardiac delivery of 1.0 μg/ml SDF-1 into infarcted hearts ($p < 0.01$) (results are expressed as mean + sem of three separate experiments).

10   (E) shows that at 2 weeks the numbers of apoptotic myocytes at the peri-infarct rim, defined by concomitant staining with anti-desmin mAb and DNA end-labeling using TUNEL technique, are significantly reduced in rats receiving either 2.0 x 10^5 endothelial progenitor cells or 10^5

15   endothelial progenitor cells together with anti-CXCR4 mAb in comparison to rats receiving 10^3 or 10^5 endothelial progenitor cells ($p < 0.01$) (results are expressed as mean + sem of three separate experiments).

       (F) shows that co-administration of anti-CXCR4 mAb or

20   intracardiac injection of SDF-1 resulted in further reductions in cardiomyocyte apoptosis of 65% and 76%, respectively, at two weeks (both $p < 0.001$) (results are expressed as mean + sem of three separate experiments).

25   __Figures 4A-4H__. Infarct Bed Vasculogenesis Improves Long-Term Myocardial Function Through Mechanisms Involving B o t h   C a r d i o m y o c y t e   P r o t e c t i o n   a n d Proliferation/Regeneration.

       (A) and (B) show the relationship between the number of

30   human CD117^{bright} endothelial progenitor cells (angioblasts) injected intravenously (10^3, 10^5, 10^5 plus anti-CXCR4 mAb, and 2×10^5) and improvement in myocardial function at 15 weeks, defined as mean improvement in left ventricular ejection fraction (LVEF) (A) and mean reduction in left

35   ventricular area at end-systole (LVAs) (B). No significant

improvement in these parameters was observed in the groups receiving 10^3 or 10^5 endothelial progenitor cells in comparison to rats receiving saline alone. In contrast, rats receiving 10^5 endothelial progenitor cells plus

5   anti-CXCR4 mAb demonstrated significant recovery in LVEF and reduction in LVAs (both p<0.001). The group receiving 2×10^5 endothelial progenitor cells demonstrated still 50% greater recovery in LVEF and reduction in LVAs (both p<0.001).

10   (C) Section from infarct of representative animal receiving 2×10^5 endothelial progenitor cells (angioblasts) showing a high frequency of cardiomyocytes staining positively for both cardiac-specific troponin I and rat-specific Ki-67 (arrows). Note the proximity of Ki-67-positive

15   cardiomyocytes to capillaries (arrowheads).
        (D) Section from infarct of representative animal receiving 2×10^5 endothelial progenitor cells showing "finger" of cardiomyocytes of rat origin, as determined by expression of rat MHC class I molecules, extending from the

20   peri-infarct region into the infarct zone. These cellular islands contain a high frequency of myocytes staining positively for both cardiac-specific troponin I and rat-specific Ki-67 (arrows). Sections from infarcts of representative animals receiving saline do not show same

25   frequency of dual staining myocytes. Bar graph shows that the group of animals receiving 2×10^5 human endothelial progenitor cells has a significantly higher index of cell cycling cardiomyocytes at the peri-infarct region than saline controls or sham operated animals (both p<0.01). No

30   difference between the groups is seen at sites distal to the infarct.
        (E) shows that the index of cell-cycling cardiomyocytes at the peri-infarct rim was increased by a further 1.9-fold when 2×10^5 human endothelial progenitor cells were

35   intravenously co-administered together with SDF-1 injected

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directly into the ischemic myocardium alone (p<0.01), or an 8-fold cumulative increase in cell-cycling cardiomyocytes at two weeks compared with LAD-ligated controls receiving saline (results are expressed as mean + sem of three
5   separate experiments).

(F) shows that intravenous co-administration of anti-CXCR4 mAb, or intracardiac co-administration of SDF-1, but not IL-8, results in 2.8 to 4-fold greater LVEF improvement, determined by echocardiography, compared with intravenous
10   injection of $2x10^5$ endothelial progenitor cells alone (p<0.01) (results are expressed as mean + sem of three separate experiments).

(G) shows that at 15 weeks the mean proportion of scar/normal left ventricular myocardium in rats receiving
15   either or 10^5 endothelial progenitor cells together with anti-CXCR4 mAb was significantly reduced in comparison to rats receiving either 10^3 or 10^5 endothelial progenitor cells (angioblasts) alone, or saline (p<0.01). The group receiving 2.0×10^5 endothelial progenitor cells
20   demonstrated still 38% greater reduction in the ratio of scar/muscle tissue (results are expressed as mean + sem of three separate experiments).

(H) Sections of rat hearts stained with Masson's trichrome at 15 weeks after LAD ligation and injection of 2.0×10^6
25   G-CSF mobilized human cells containing 10^3 (left) or 2.0×10^5 (right) CD117^{bright} endothelial progenitor cells. Hearts of rats receiving 10^3 endothelial progenitor cells had greater loss of anterior wall mass, collagen deposition (lighter gray), and septal hypertrophy compared with hearts
30   of rats receiving 2.0×10^5 endothelial progenitor cells.

Figure 5: This figure shows mRNA expression of three genes in the ischemic rat hearts at various time points. You will
35   see that at 48 hours and 2 weeks after LAD ligation and

ischemia, HBP23 (the rat homologue of human PAG/NKEF-A,B,C,
all of which are part of the family of peroxiredoxins
(Prx)) is decreased, and vitamin D3 upregulated protein
VDUP-1 is increased. The early (48 hour) reduction in PRX
5   and increase in VDUP-1 results in a compensatory increase
in thiol reductase thioredoxin (TRX). Note that endothelial
progenitor cell therapy reverses this pattern of mRNA
expression.

10   Figure 6: DNA sequence (SEQ ID NO:1) corresponding to mRNA
encoding VDUP-1.

Figure 7: This figure shows catalytic DNA 5'-AT-3' cleavage
sites on VDUP-1 DNA (SEQ ID NO:3) corresponding to the
15   coding region of VDUP-1 mRNA. Cleavage site pairs are
uppercase.

Figure 8: This figure shows catalytic DNA 5'-GC-3' cleavage
sites on VDUP-1 DNA (SEQ ID NO:3) corresponding to the
20   VDUP-1 mRNA. Cleavage site pairs are uppercase.

Figure 9: This figure shows catalytic DNA 5'-GT-3' cleavage
sites on VDUP-1 DNA (SEQ ID NO:3) corresponding to the
VDUP-1 mRNA. Cleavage site pairs are uppercase.
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Figure 10: This figure shows catalytic DNA 5'-AC-3'
cleavage sites on VDUP-1 DNA (SEQ ID NO:3) corresponding to
the VDUP-1 mRNA. Cleavage site pairs are uppercase.

30   Figure 11: This figure shows sites which can be cleaved by
a hammerhead ribozyme in VDUP-1 DNA (SEQ ID NO:3)
corresponding to the VDUP-1 mRNA coding region. Uppercase
"T" represents cleavage site.

35   Figure 12: This figure shows that at VDUP-1 enzyme

concentrations ranging from 0.05μM to 5μM the sequence-specific VDUP1 DNA enzyme cleaved a synthetic rat VDUP1 oligonucleotide in a concentration- and time-dependent manner.

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Figure 13: (a) Shows intramyocardial injection of the rat sequence-specific VDUP1 DNA enzyme at 48 hours after LAD ligation resulted in a 75% mean inhibition of proliferating cardiac fibroblasts in the infarct zone two weeks later in 10 comparison to injection of scrambled DNA enzyme control (p<0.01). (b) Shows injection of VDUP1 DNA enzyme resulted in 20% mean reduction in apoptotic cardiomyocytes at the peri-infarct region relative to injection with the scrambled DNA enzyme control (p<0.05).

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Figure 14: (a) Shows inhibition of fibroblast proliferation and cardiomyocyte apoptosis resulted in significant reduction of mature scar deposition in the infarct zone, from a mean of 35% for animals receiving control scrambled 20 DNA enzyme to a mean of 20% for those receiving VDUP1 DNA enzyme (p<0.01). (b) Shows animals receiving VDUP1 DNA enzyme demonstrated a 50% mean recovery in cardiac function, as determined by ejection fraction, whereas no improvement was seen in animals receiving scrambled control 25 DNA enzyme (p<0.01)

Figure 15: (a) Shows that G-CSF, when used systematically at the same dosages after myocardial infarction, is a more potent inducer of cardiac neovascularization than GM-CSF. 30 (B) Shows G-CSF is a more potent inhibitor of cardiomyocyte apoptosis than GM-CSF.

Figure 16: (a) Shows G-CSF is a more potent inducer of cardiomyocyte·regeneration than GM-CSF, and (b) shows that 35 G-CSF enables significantly greater recovery of cardiac

function after acute myocardial infarction.

Figure 17: (a) Shows intravenous administration of anti-CXCR4 monoclonal antibody after acute myocardial infarction induces significantly greater numbers of blood vessels at the peri-infarct region. (b) Shows the greater recovery of cardiac/myocardial function than with control antibodies.

Figure 18: Shows myocardial expression of SDF-1 mRNA increased by two weeks, but not within the first 48 hours after myocardial infarction, and is inhibited by subcutaneously injecting GM-CSF.

Figure 19: (a) Shows intramyocardial injection of SDF-1 induces neovascularization at the peri-infarct region and (b) protects cardiomyocytes at the peri-infarct region against apoptosis.

Figure 20: (a) Shows intramyocardial administration of SDF-1 alone, and synergistically with GM-CSF induces cardiomyocyte regeneration at the peri-infarct region, and (b) improves cardiac function.

Figure 21: (a) Shows Human angioblasts, or endothelial progenitor cells, induce neovascularization as early as 5 days post-infarct. (b) Shows Human angioblasts, or endothelial progenitor cells protect peri-infarct cardiomyocytes against apoptosis as early as 5 days post infarct.

Figure 22: (a) Shows animals receiving human bone marrow-derived CD34+ cells demonstrated numerous clusters of small, cycling cells at the peri-infarct region that were of rat origin, as defined by a monoclonal antibody specific for rat Ki67. (b) Shows tissues obtained from

animals sacrificed at two weeks after human CD34+ administration no longer demonstrated clusters of small, cycling cardiomyocyte progenitors, but instead a high frequency of large, mature rat cardiomyocytes at the peri-infarct region with detectable DNA activity, as determined by dual staining with mAbs reactive against cardiomyocyte-specific troponin I and mAbs reactive against rat Ki67. (c) Shows cycling by the same staining.

Figure 23: Shows by RT-PCR, HBP23 mRNA levels in rat hearts decreased at two weeks post-LAD ligation by a mean of 34% compared with normal rat hearts.

Figure 24: (a) Shows a DNA enzyme against HBP23 cleaved the 23-base oligonucleotide synthesized from the sequence of rat HBP23 mRNA, in a dose- and time-dependent manner. (b) Shows densitometric analysis of RT-PCR products following reverse transcription of cellular mRNA, demonstrating the HBP23 DNA enzyme inhibited steady-state mRNA levels in cultured rat cells by over 80% relative to the scrambled DNA.

Figure 25: (a) Shows HBP23 DNA enzyme had no effect on induction of neovascularization by human bone marrow angioblasts, no increased myocardial capillary density in comparison to saline controls is seen. (b) Shows injection of the HBP23 DNA enzyme, but not the scrambled control, abrogated the anti-apoptotic effects of neovascularization. (c) Shows injection of the HBP23 DNA enzyme, but not the scrambled control, abrogated the improvement in cardiac function.

Detailed Description

As used herein, "VEGF" is defined as vascular endothelial growth factor. "VEGF-R" is defined as vascular endothelial
5   growth factor receptor. "FGF" is defined as fibroblast growth factor. "IGF" is defined as Insulin-like growth factor. "SCF" is defined as stem cell factor. "G-CSF" is defined as granulocyte colony stimulating factor. "M-CSF" is defined as macrophage colony stimulating factor. "GM-
10   CSF" is defined as granulocyte-macrophage colony stimulating factor. "MCP" is defined as monocyte chemoattractant protein.

As used herein "angioblasts" is synonymous with the term
15   "endothelial progenitor cells".

As used herein, "CXC" chemokine refers to the structure of the chemokine. Each "C" represents a cysteine and "X" represents any amino acid. "CXCR4" refers to CXC receptor
20   4.

As used herein, "CC" chemokine refers to the structure of the chemokine. Each "C" represents a cysteine.

25   A "cardiac progenitor cell" refers to a cell that is resident in the heart, or that comes into the heart from elsewhere after acute ischemia, is smaller than mature cardiomyocytes, expresses alpha sarcomeric actin but is negative for troponin, is normally quiescent but can be
30   induced to go into cell cycle as defined by positive Ki67 staining. "Cardiac progenitor cell" may be used synonymously with "cardiomyocyte progenitor cell".

"Catalytic" shall mean the functioning of an agent as a
35   catalyst, i.e. an agent that increases the rate of a

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chemical reaction without itself undergoing a permanent structural change.

"Nucleic acid" shall include without limitation any nucleic
5   acid, including, without limitation, DNA, RNA, oligonucleotides, or polynucleotides, and analogs or derivatives thereof. The nucleotides that form the nucleic acid may be nucleotide analogs or derivatives thereof. The nucleic acid may incorporate non nucleotides.
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"Nucleotides" shall include without limitation nucleotides and analogs or derivatives thereof. For example, nucleotides may comprise the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are
15   well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New Jersey, USA).

20   "Proliferation" and "regeneration" are used synonymously when referring to cardiomyocytes in this application. "Proliferation" in respect to cardiomyocytes, shall mean a fold increase in proportion of cardiomyocytes entering the cell cycle relative to untreated rat heart.
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"Trafficking" means the blood-borne migration of cells, in particular angioblast/endothelial progenitor cells.

This invention provides a method of treating a disorder of
30   a subject's heart involving loss of cardiomyocytes which comprises administering to the subject an amount of an agent effective to cause cardiomyocyte proliferation within the subject's heart so as to thereby treat the disorder.

35   In one embodiment the agent is human endothelial progenitor

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cells. In one embodiment the endothelial progenitor cells are bone marrow-derived. In another they are derived from cord blood, or embryonic or fetal sources. Effective amounts of endothelial progenitor cells sufficient to cause cardiomyocyte proliferation can be done based on animal data using routine computational methods. In one embodiment the effective amount is about $1.5x10^5$ endothelial progenitor cells per kg body mass to about $3x10^5$ per kg body mass. In another embodiment the effective amount is about $3x10^5$ per kg body mass to about $4.5x10^5$ endothelial progenitor cells per kg body mass. In another embodiment the effective amount is about $4.5x10^5$ per kg body mass to about $5.5x10^5$ endothelial progenitor cells per kg body mass. In another embodiment the effective amount is about $5.5x10^5$ per kg body mass to about $7x10^5$ endothelial progenitor cells per kg body mass. In another embodiment the effective amount is about $7x10^5$ per kg body mass to about $1x10^6$ endothelial progenitor cells per kg body mass. In another embodiment the effective amount is about $1x10^6$ per kg body mass to about $1.5x10^6$ endothelial progenitor cells per kg body mass. In one embodiment the effective amount of human endothelial progenitor cells is between about $1.5x10^6$ and $4.5x10^6$ endothelial progenitor cells per kg of the subject's body mass and In a preferred embodiment the effective amount is about $5x10^5$ endothelial progenitor cells per kg of the subject's body mass.

In one embodiment the endothelial progenitor cells are allogeneic with respect to the subject. In differing embodiments the subject is an adult or an embryo or a fetus. In another embodiment the endothelial progenitor cells are derived from cloned autologous embryonic stem cells.

In one embodiment the agent induces expression of a mRNA

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encoding a peroxiredoxin. The expression of peroxiredoxin mRNA may be increased, for example, by administration of 2(3)-t-butyl-4-hydroxyanisole (BHA) (see 106) which has been shown to increase expression of Peroxiredoxin-1 when

5   administered by diet. Alternatively, local control of O_2 can have the same effect (see 107). Peroxiredoxin mRNA expression, such as that of thiol peroxidases, may also be induced by heme, cadmium or cobalt (see 108). Peroxiredoxins include, but are not limited to, PAG, HBP23,

10   MSP23, NKEF.

In another embodiment the agent induces expression of a mRNA encoding NF-E2-related factor 2 (Nrf2). Cytomplasmic NF-E2-related factor 2 (Nrf2) expression can be indirectly

15   increased by raising free Nrf2 levels. Since Nrf2 is tightly bound to keap1 in the cytoplasm then reducing expression of keap1 mRNA is a suitable target e.g. by keap1 antisense oligonucleotides or catalytic nucleic acids (see 109 for keap1). Also, blocking the interaction between Nrf2

20   and Keap1 by inhibiting the interaction of the Neh2 domain of Nrf2 and the DGR domain of keap1, e.g. by using the entire Neh2 domain of nrf2, amino acids 1-73, or only the hydrophilic region, amino acids 33-73 (see 109) will increase free cytoplasmic Nrf-2. In another embodiment the

25   agent induces dissociation of a Nrf2 protein from a Keap-1. In another embodiment the agent inhibits association of a Nrf2 protein with a Keap-1. In another embodiment the agent inhibits association of a thiol reductase thioredoxin with a VDUP-1 protein. In another embodiment the agent inhibits

30   c-Abl tyrosine kinase activation. In a further embodiment the agent is STI-571.

In one embodiment the agent is a CXC chemokine. In further embodiments the agent is Stromal-Derived Factor-1, Il-8 or

35   Gro-Alpha. In one embodiment the amount of CXC chemokine

administered is between 0.2 and 5µg/ml at a max volume of 10ml for a 70kg human subject. In a preferred embodiment the amount is about 1µg/ml. In one embodiment the agent is an inhibitor of plasminogen activator inhibitor-1. In

5   another embodiment the agent is an antibody directed against an epitope of CXCR4. In one embodiment the amount of antibody directed against an epitope of CXCR4 is between 25 and 75µg/ml, at a max volume of 10ml for a 70kg human subject. A simple calculation is performed for subjects of

10   different mass. In a preferred embodiment the amount is about 50µg/ml.

Fin differring embodiments the agent is Stromal-Derived Factor-1 alpha or Stromal-Derived Factor-1 beta. In differing embodiments the chemokine can be administered

15   intramyocardially, intracoronary, and/or via a stent, a scaffold, or as a slow-release formulation.

In differing embodiments the agent is G-CSF, GM-CSF, or a Gro family chemokine. In a further embodiment the agent is

20   Gro alpha.

In a further embodiment the instant method further comprises administering an effective amount of a second agent that increases the cardiomyocyte proliferation caused

25   by the human endothelial progenitor cells. Effective amounts of the second agent are amounts sufficient to enhance or accelerate cardiomyocyte proliferation in the presence of administered endothelial progenitor cells. In further embodiments the endothelial progenitor cells

30   express CD117, CD34, AC133 or a high level of intracellular GATA-2 activity. In one embodiment the administering comprises injecting directly into the subject's peripheral circulation, heart muscle, left ventricle, right ventricle, coronary artery, cerebro-spinal fluid, neural tissue,

35   ischemic tissue, or post-ischemic tissue.

In one embodiment the second agent is an antisense oligonucleotide which specifically inhibits translation of Vitamin D3 Up-Regulated Protein-1 (VDUP-1) mRNA.

5   Therapeutically useful targeted inhibition of VDUP-1 protein (SEQ ID NO:2) expression can be achieved through the use of antisense oligonucleotides. Antisense oligonucleotides are small fragments of DNA and derivatives thereof complementary to a defined sequence on a specified
10   mRNA. A VDUP-1 antisense oligonucleotide specifically binds to targets on the VDUP-1 mRNA (SEQ ID NO:1) molecule and in doing so inhibits the translation thereof into VDUP-1 protein (SEQ ID NO:2).

15   Antisense oligonucleotide molecules synthesized with a phosphorothioate backbone have proven particularly resistant to exonuclease damage compared to standard deoxyribonucleic acids, and so they are used in preference. A phosphorothioate antisense oligonucleotide for VDUP-1
20   mRNA can be synthesized on an Applied Biosystems (Foster City, CA) model 380B DNA synthesizer by standard methods. E.g. Sulfurization can be performed using tetraethylthiuram disulfide/acetonitrile. Following cleavage from controlled pore glass support, oligodeoxynucleotides can be base
25   deblocked in ammonium hydroxide at 60°C for 8 h and purified by reversed-phase HPLC [0.1M triethylammonium bicarbonate /acetonitrile; PRP-1 support]. Oligomers can be detritylated in 3% acetic acid and precipitated with 2% lithiumperchlorate/acetone, dissolved in sterile water and
30   reprecipitated as the sodium salt from 1 M NaCl/ethanol. Concentrations of the full length species can be determined by UV spectroscopy.

Any other means for such synthesis known in the art may
35   additionally or alternatively be employed. It is well known

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to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

Hybridization of antisense oligonucleotides with VDUP-1 mRNA interferes with one or more of the normal functions of VDUP-1 1 mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Also, oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof) are included. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages

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include, but are not limited to, U.S. Pat. Nos. 3,687,808;
4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897;
5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131;
5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;
5 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111;
5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555;
5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of
which are commonly owned with this application, and each of
which is herein incorporated by reference.

10

In accordance with this invention, persons of ordinary
skill in the art will understand that messenger RNA
includes not only the information to encode a protein using
the three letter genetic code, but also associated
15   ribonucleotides which form a region known to such persons
as the 5'-untranslated region, the 3'-untranslated region,
the 5' cap region and intron/exon junction ribonucleotides.
Thus, antisense oligonucleotides or the catalytic nucleic
acids described below may be formulated in accordance with
20   this invention which are targeted wholly or in part to
these associated ribonucleotides as well as to the
informational ribonucleotides.   The   antisense
oligonucleotides may therefore be specifically hybridizable
with a transcription initiation site region, a translation
25   initiation codon region, a 5' cap region, an intron/exon
junction, coding sequences, a translation termination codon
region or sequences in the 5'- or 3'-untranslated region.
Similarly, the catalytic nucleic acids may specifically
cleave a   transcription   initiation   site   region, a
30   translation initiation codon region, a 5' cap region, an
intron/exon junction, coding sequences, a translation
termination codon region or sequences in the 5'- or 3'-
untranslated region. As is known in the art, the
translation initiation codon is typically 5'-AUG (in
35   transcribed mRNA molecules; 5'-ATG in the corresponding DNA

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molecule). A minority of genes have a translation
initiation codon having the RNA sequence 5'-GUG, 5'UUG or
5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to
function in vivo. Thus, the term "translation initiation
5   codon" can encompass many codon sequences, even though the
initiator amino acid in each instance is typically
methionine in eukaryotes. It is also known in the art that
eukaryotic genes may have two or more alternative
translation initiation codons, any one of which may be
10   preferentially utilized for translation initiation in a
particular cell type or tissue, or under a particular set
of conditions. In the context of the invention,
"translation initiation codon" refers to the codon or
codons that are used in vivo to initiate translation of an
15   mRNA molecule transcribed from a gene encoding VDUP-1,
regardless of the sequence(s) of such codons. It is also
known in the art that a translation termination codon of a
gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG
and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-
20   TAG and 5'-TGA, respectively). The term "translation
initiation codon region" refers to a portion of such an
mRNA or gene that encompasses from about 25 to about 50
contiguous nucleotides in either direction (i.e., 5' or 3')
from a translation initiation codon. This region is one
25   preferred target region. Similarly, the term "translation
termination codon region" refers to a portion of such an
mRNA or gene that encompasses from about 25 to about 50
contiguous nucleotides in either direction (i.e., 5' or 3')
from a translation termination codon. This region is also
30   one preferred target region. The open reading frame or
"coding region," which is known in the art to refer to the
region between the translation initiation codon and the
translation termination codon, is also a region which may
be targeted effectively. Other preferred target regions
35   include the 5' untranslated region (5'UTR), known in the

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art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. mRNA splice sites may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions may also be preferred targets.

Once the target site or sites have been identified, antisense oligonucleotides can be chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired disruption of the function of the molecule. "Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the catalytic nucleic acids. Similarly, catalytic nucleic acids are synthesized once cleavage target sites on the VDUP-1 mRNA molecule.

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It is preferred to administer antisense oligonucleotides or catalytic nucleic acids or analogs thereof or other agents to mammals suffering from cardiovascular disease, in either native form or suspended in a carrier medium in amounts and upon treatment schedules which are effective to therapeutically treat the mammals to reduce the detrimental effects of cardiovascular disease. One or more different catalytic nucleic acids or antisense oligonucleotides or analogs thereof targeting different sections of the nucleic acid sequence of VDUP-1 mRNA may be administered together in a single dose or in different doses and at different amounts and times depending upon the desired therapy. The catalytic nucleic acids or antisense oligonucleotides can be administered to mammals in a manner capable of getting the oligonucleotides initially into the blood stream and subsequently into cells, or alternatively in a manner so as to directly introduce the catalytic nucleic acids or antisense oligonucleotides into the cells or groups of cells, for example cardiomyocytes, by such means by electroporation or by direct injection into the heart. Antisense oligonucleotides whose presence in cells can inhibit transcription or protein synthesis can be administered by intravenous injection, intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, orally or rectally. Human pharmacokinetics of certain antisense oligonucleotides have been studied. See (105) incorporated by reference in its entirety. It is within the scale of a person's skill in the art to determine optimum dosages and treatment schedules for such treatment regimens.

Any of the agents employed in this invention can be administered intracoronary or intramyocardially, for

example by injection. The agents can be delivered in various means as otherwise discussed, including by stents, such as drug-eluting stents, and in time-release formats such as slow-release. Where the agents are proteins,

5   including but not limited to SDF-1, G-CSF, GM-CSF, and VEGF, the agents can be administered directly or indirectly, or may be administered via induction of gene expression, through gene therapy, adenoviral delivery and other such methods familiar to those skilled in the art.

10

Doses of the oligonucleotides or analogs thereof of the present invention in a pharmaceutical dosage unit will be an efficacious, nontoxic quantity administered to a human patient in need of cardiomyocyte regeneration (or

15 inhibition of VDUP-1 expression) from 1-6 or more times daily or every other day. Dosage is dependent on severity and responsiveness of the effects of abnormal cardiovascular disease to be treated, with course of treatment lasting from several days to months or until a

20 cure is effected or a reduction of the effects is achieved. Oral dosage units for human administration generally use lower doses. The actual dosage administered may take into account the size and weight of the patient, whether the nature of the treatment is prophylactic or therapeutic in

25 nature, the age, weight, health and sex of the patient, the route of administration, and other factors.

In another embodiment the second agent is a pro-angiogenic agent. In further embodiments the pro-angiogenic agent is

30 vascular endothelial growth factor, fibroblast growth factor or angiopoietin. In another embodiment the second agent induces expression of a pro-angiogenic factor. In a further embodiment the second agent is Hypoxia Inducible Factor-1.

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In another embodiment the second agent is a catalytic nucleic acid which specifically inhibits translation of Vitamin D3 Up-Regulated Protein-1 mRNA. In a further

5 embodiment the catalytic nucleic acid comprises deoxyribonucleotides. In another embodiment the catalytic nucleic acid comprises ribonucleotides.

Catalytic nucleic acid molecules can cleave Vitamin D3

10 Up-Regulated Protein-1 (VDUP-1) mRNA (corresponding DNA shown in SEQ ID NO:1, Figure 5) at each and any of the consensus sequences therein. Since catalytic ribo- and deoxyribo- nucleic acid consensus sequences are known, and the VDUP-1 Protein mRNA sequence is known, one of ordinary

15 skill could readily construct a catalytic ribo- or deoxyribo nucleic acid molecule directed to any of the VDUP-1 protein mRNA consensus sequences based on the instant specification. In preferred embodiments of this invention the catalytic deoxyribonucleic acids include the

20 10-23 structure. Examples of catalytic ribonucleic acids include hairpin and hammerhead ribozymes. In preferred embodiments of this invention, the catalytic ribonucleic acid molecule is formed in a hammerhead (50) or hairpin motif (51,52,53), but may also be formed in the motif of a

25 hepatitis delta virus (54), group I intron (60), RNaseP RNA (in association with an RNA guide sequence) (55,56) or Neurospora VS RNA (57,58,59).

To target the VDUP-1 mRNA (SEQ ID NO:1), catalytic nucleic

30 acids can be designed based on the consensus cleavage sites 5'-purine:pyrimidine-3' in the VDUP-1 mRNA sequence (104) (see figures 6-10) for cleavage sites on DNA corresponding to the mRNA encoding VDUP-1 (SEQ ID NO:2). Those potential

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cleavage sites located on an open loop of the mRNA
according to RNA folding software e.g. RNADRaw 2.1 are
particularly preferred as targets (61). The DNA based
catalytic nucleic acids can utilize the structure where two
5   sequence-specific arms are attached to a catalytic core
based on the VDUP-1 mRNA sequence. Further examples of
catalytic DNA structure are detailed in (62) and (63).
Commercially available mouse brain polyA-RNA (Ambion) can
serve as a template in the *in vitro* cleavage reaction to
10   test the efficiency of the catalytic deoxyribonucleic
acids. Catalytic RNA, is described above, is designed
similarly. Hammerhead ribozymes can cleave any 5'-NUH-3'
triplets of a mRNA, where U is conserved and N is any
nucleotide and H can be C,U,A, but not G. For example, the
15   sites which can be cleaved by a hammerhead ribozyme in
human VDUP-1 mRNA coding region are shown in figure 10.

Cleaving of VDUP-1 mRNA with catalytic nucleic acids
interferes with one or more of the normal functions of
20   VDUP-1 mRNA. The functions of mRNA to be interfered with
include all vital functions such as, for example,
translocation of the RNA to the site of protein
translation, translation of protein from the RNA, splicing
of the RNA to yield one or more mRNA species, and catalytic
25   activity which may be engaged in by the RNA.

The nucleotides may comprise other bases such as inosine,
deoxyinosine, hypoxanthine may be used. In addition,
isoteric   purine   2'deoxy-furanoside   analogs,
30   2'-deoxynebularine or 2'deoxyxanthosine, or other purine or
pyrimidine analogs may also be used. By carefully selecting
the bases and base analogs, one may fine tune the
hybridization properties of the oligonucleotide. For

example, inosine may be used to reduce hybridization specificity, while diaminopurines may be used to increase hybridization specificity.

5   Adenine and guanine may be modified at positions N3, N7, N9, C2, C4, C5, C6, or C8 and still maintain their hydrogen bonding abilities. Cytosine, thymine and uracil may be modified at positions N1, C2, C4, C5, or C6 and still maintain their hydrogen bonding abilities. Some base

10   analogs have different hydrogen bonding attributes than the naturally occurring bases. For example, 2-amino-2'-dA forms three (3), instead of the usual two (2), hydrogen bonds to thymine (T). Examples of base analogs that have been shown to increase duplex stability include, but are not limited

15   to, 5-fluoro-2'-dU, 5-bromo-2'-dU, 5-methyl-2'-dC, 5-propynyl-2'-dC, 5-propynyl-2'-dU, 2-amino-2'-dA, 7-deazaguanosine, 7-deazadenosine, and N2-Imidazoylpropyl-2'-dG.

20   Nucleotide analogs may be created by modifying and/or replacing a sugar moiety. The sugar moieties of the nucleotides may also be modified by the addition of one or more substituents. For example, one or more of the sugar moieties may contain one or more of the following

25   substituents: amino, alkylamino, araalkyl, heteroalkyl, heterocycloalkyl, aminoalkylamino, O, H, an alkyl, polyalkylamino, substituted silyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O- alkyl, S-alkyl, SOMe, SO_2Me, ONO_2, NH-alkyl, $OCH_2CH=CH_2$, OCH_2CCH, OCCHO, allyl, O-allyl, NO_2, N_3, and NH_2.

30   For example, the 2' position of the sugar may be modified to contain one of the following groups: H, OH, OCN, O-alkyl, F, CN, CF_3, allyl, O-allyl, OCF_3, S- alkyl, SOMe, SO_2Me, ONO_2, NO_2, N_3, NH_2, NH-alkyl, or $OCH=CH_2$, OCCH, wherein

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the alkyl may be straight, branched, saturated, or unsaturated. In addition, the nucleotide may have one or more of its sugars modified and/or replaced so as to be a ribose or hexose (i.e. glucose, galactose) or have one or

5   more anomeric sugars. The nucleotide may also have one or more L-sugars.

Representative United States patents that teach the preparation of such modified bases/nucleosides/nucleotides

10   include, but are not limited to, U.S. Pat. Nos. 6,248,878, and 6,251,666 which are herein incorporated by reference.

The sugar may be modified to contain one or more linkers for attachment to other chemicals such as fluorescent

15   labels. In an embodiment, the sugar is linked to one or more aminoalkyloxy linkers. In another embodiment, the sugar contains one or more alkylamino linkers. Aminoalkyloxy and alkylamino linkers may be attached to biotin, cholic acid, fluorescein, or other chemical

20   moieties through their amino group.

Nucleotide analogs or derivatives may have pendant groups attached. Pendant groups serve a variety of purposes which include, but are not limited to, increasing cellular uptake

25   of the oligonucleotide, enhancing degradation of the target nucleic acid, and increasing hybridization affinity. Pendant groups can be linked to any portion of the oligonucleotide but are commonly linked to the end(s) of the oligonucleotide chain. Examples of pendant groups

30   include, but are not limited to: acridine derivatives (i.e. 2-methoxy-6-chloro-9- aminoacridine); cross-linkers such as psoralen derivatives, azidophenacyl, proflavin, and azidoproflavin; artificial endonucleases; metal complexes

such as EDTA-Fe(II), o-phenanthroline-Cu(I), and porphyrin-Fe(II); alkylating moieties; nucleases such as amino-1-hexanolstaphylococcal nuclease and alkaline phosphatase; terminal transferases; abzymes; cholesteryl

5   moieties; lipophilic carriers; peptide conjugates; long chain alcohols; phosphate esters; amino; mercapto groups; radioactive markers; nonradioactive markers such as dyes; and polylysine or other polyamines. In one example, the nucleic acid comprises an oligonucleotide conjugated to a

10   carbohydrate, sulfated carbohydrate, or gylcan. Conjugates may be regarded as a way as to introduce a specificity into otherwise unspecific DNA binding molecules by covalently linking them to a selectively hybridizing oligonucleotide.

15   The catalytic nucleic acid binding domains (i.e. the non-catalytic domains) or antisense oligonucleotide may comprise modified bonds. For example internucleosides bonds of the oligonucleotide may comprise phosphorothioate linkages. The nucleic acid may comprise nucleotides having

20   moiety may be modified by replacing one or both of the two bridging oxygen atoms of the linkage with analogues such as -NH, -CH$_2$, or -S. Other oxygen analogues known in the art may also be used. The phosphorothioate bonds may be stereo regular or stereo random.

25

The oligonucleotide moiety may have one or more of its sugars modified or replaced so as to be ribose, glucose, sucrose, or galactose, or any other sugar. Alternatively, the phosphorothioate oligonucleotide may have one or more

30   of its sugars substituted or modified in its 2' position, i.e. 2'allyl or 2'-O-allyl. An example of a 2'-O-allyl sugar is a 2'-O-methylribonucleotide. Further, the phosphorothioate oligonucleotide may have one or more of

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its sugars substituted or modified to form an α-anomeric
sugar.

5   A catalytic nucleic acid may include non-nucleotide
substitution. The non-nucleotide substitution includes
either abasic nucleotide, polyether, polyamine, polyamide,
peptide, carbohydrate, lipid or polyhydrocarbon compounds.
The term "abasic" or "abasic nucleotide" as used herein
encompasses sugar moieties lacking a base or having other
10   chemical groups in place of base at the 1' position.

Determining the effective amount of the instant nucleic
acid molecules can be done based on animal data using
routine computational methods. In one embodiment, the
15   effective amount contains between about 10 ng and about 100
μg of the instant nucleic acid molecules per kg body mass.
In another embodiment, the effective amount contains
between about 100 ng and about 10 μg of the nucleic acid
molecules per kg body mass. In a further embodiment, the
20   effective amount contains between about 1 μg and about 5
μg, and in a further embodiment about 2 μg, of the nucleic
acid molecules per kg body mass.

In another embodiment the second agent is cardiomyocyte
25   progenitor cells. In another embodiment the second agent is
skeletal muscle progenitor cells. Either of theses cell
types can be derived from embryonic, fetal or adult
subjects.

30   In one embodiment the second agent promotes trafficking of
the endothelial progenitor cells to the subject's heart.

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In a further embodiment the second agent that promotes trafficking is an antibody directed against an epitope of CXCR4.

5   In another further embodiment the second agent that promotes trafficking is a CC chemokine. In further embodiments the CC chemokine is RANTES, EOTAXIN, monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, or MCP.

10   In another further embodiment the second agent that promotes trafficking is a CXC chemokine. In further embodiments the CXC chemokine is Interleukin-8, Gro-Alpha, or Stromal-Derived Factor-1.

15   In one embodiment the second agent promotes mobilization of angioblasts into the subject's bloodstream. In different embodiments the agent is G-CSF, GM-CSF, SDF-1, or VEGF.

This invention also provides a method of determining the
20   susceptibility of a cardiomyocyte in a subject to apoptosis comprising:

    (a)   quantitating the amount of a mRNA encoding peroxiredoxin in the cardiomyocyte;

    (b)   quantitating the amount of a mRNA encoding
25   Vitamin D3 Up-Regulated Protein-1 in the cardiomyocyte; and

    (c)   determining the ratio of the amount of mRNA encoding peroxiredoxin: amount of mRNA encoding Vitamin D3 Up-Regulated Protein-1, wherein a low
30   ratio indicates a high susceptibility of the cardiomyocyte to apoptosis and a high ratio

indicates a low susceptibility of the cardiomyocyte to apoptosis in the subject.

This invention also provides a method of determining the susceptibility of a cardiomyocyte in a subject to apoptosis comprising:

(a) quantitating the expression of a peroxiredoxin protein in the cardiomyocyte;

(b) quantitating the expression of Vitamin D3 Up-Regulated Protein-1 in the cardiomyocyte; and

(c) determining the ratio of the peroxiredoxin protein expression: Vitamin D3 Up-Regulated Protein-1 expression, wherein a low ratio indicates a high susceptibility of the cardiomyocyte to apoptosis and a high ratio indicates a low susceptibility of the cardiomyocyte to apoptosis in the subject.

A ratio that is at least two standard deviations above that found in control patients with normal hearts is considered a high ratio. Quantitation of expression of protein in cardiomyocytes is performed using routine methods known to those of skill in the art, for example, northern and western blots.

This invention further provides a cardiac progenitor cell. In an embodiment the cardiac progenitor cell is resident in the heart, or that comes into the heart from elsewhere after acute ischemia, is smaller than mature cardiomyocytes, expresses alpha sarcomeric actin but is negative for troponin, is normally quiescent but can be induced to go into cell cycle as defined by positive Ki67

staining.

This invention further provides a method of inducing cell cycling in a cardiac progenitor cell in a heart tissue of a subject comprising administering to the subject an amount of an agent effective to induce neovascularization of the heart tissue in the subject so as to thereby induce cardiac cell cycling within the heart tissue of the subject.

This invention further provides a method of improving cardiac function in a subject comprising administering to the subject an amount of an agent effective to induce neovascularization of a heart tissue in the subject so as to thereby improve cardiac function in the subject.

This invention further provides a method of improving cardiac function in a subject comprising administering to the subject an amount of an agent effective to induce proliferation of cardiomyocytes in the heart tissue in the subject so as to thereby improve cardiac function in the subject.

Cardiac function, or myocardial function, can be determined by any combination of improvement in ejection fraction, perfusion, reduction in anginal symptoms, improvement in quality of life, increased walking ability, longevity of life, reduction in heart medication, and/or prevention of heart failure, as this terms are normally understood in the art. An improvement in cardiac function in a subject is measured by determining an any of theses factors in a subject before and after treatment by any of the instant methods.

35

In one embodiment of the instant methods, the subject has suffered myocardial ischemia or myocardial infarct. In different embodiments the agent is G-CSF, GM-CSF, IL-8, a Gro family chemokine, an inhibitor of CXCR4, or an
5   inhibitor of SDF-1. In further embodiments the inhibitor of CXCR4 is a small molecule or a monoclonal antibody, the inhibitor of CXCR4 is a small molecule and is AMD 3100, the inhibitor of SDF-1 is a small molecule or a monoclonal antibody, and the Gro family chemokine is Gro alpha. In
10   another embodiment the inhibitor is AMD 070. In differing embodiments the the inhibitor of CXCR4 is a catalytic nucleic acid, an oligonucleotide, RNAi, (RNA interference) or a small molecule.

15   This invention further provides a method of inducing apoptosis in a cell comprising inhibiting expression of a peroxiredoxin in the cell. In one embodiment the cell is a tumor cell. In different embodiments the peroxiredoxin is peroxiredoxin I, II, II, IV, or V. In one embodiment the
20   inhibition of expression of peroxiredoxin is effected by contacting the cell with a catalytic nucleic acid which binds mRNA encoding peroxiredoxin, thereby inhibiting the expression thereof. In different embodiments the inhibition of expression of peroxiredoxin is effected by contacting
25   the cell with an antisense oligonucleotide, a monoclonal antibody, RNA interference or a small molecule.

This invention further provides a method of treating a disorder of a tissue cells of a subject involving loss of
30   tissue cells which comprises administering to the subject an amount of an agent effective to cause tissue cell proliferation within the tissue of the subject so as to thereby treat the disorder. In differing embodiments the

tissue is cardiac tissue, brain tissue, peripheral vascular tissue, hepatic tissue, renal tissue, gastrointestinal tissue, lung tissue, smooth muscle tissue, or striated muscle tissue. In differing embodiments the agent is G-CSF,

5   SDF-1, GM-CSF, IL-8, VEGF. In one embodiment the agent is an inhibitor of CXCR4/SDF-1 interaction. In further embodiments the inhibitor is a catalytic nucleic acid, a monoclonal antibody, a antisense oligonucleotide, a small molecule, or RNAi. In one embodiment the agent is an

10   inducer of peroxiredoxin expression. In further embodiments the peroxiredoxin is peroxiredoxin I, II, III, IV, or V.

In any of the instant methods wherein the agnet is G-CSF, the G-CSF may be a covalent conjugate of recombinant

15   methionyl human G-CSF (Filgrastim) and monomethoxypolyethylene glycol, such as Neulasta. Filgrastim is a water-soluble 175 amino acid protein with a molecular weight of approximately 19 kilodaltons (kd). Filgrastim is obtained from the bacterial fermentation of

20   a strain of Escherichia coli transformed with a genetically engineered plasmid containing the human G-CSF gene. To produce pegfilgrastim, a 20 kd monomethoxypolyethylene glycol molecule is covalently bound to the N-terminal methionyl residue of Filgrastim. The average molecular

25   weight of pegfilgrastim is approximately 39 kd. Neulasta is supplied in 0.6 mL prefilled syringes for subcutaneous (SC) injection. Each syringe contains 6 mg pegfilgrastim (based on protein weight), in a sterile, clear, colorless, preservative-free solution (pH 4.0) containing acetate

30   (0.35 mg), sorbitol (30.0 mg), polysorbate 20 (0.02 mg), and sodium (0.02 mg) in water for injection, USP.

This invention provides a method of treating a disorder of

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a tissue of a subject involving apoptosis of cells in the tissue which comprises administering to the subject an amount of an agent effective to inhibit apoptosis of cells in the tissue within the subject so as to thereby treat the

5   disorder. In one embodiment the agent is an inhibitor of VDUP-1 expression. In further embodiments the inhibitor of VDUP-1 expression is catalytic nucleic acid, a monoclonal antibody, an antisense oligonucleotide, a small molecule, or an RNAi. In differing embodiments the tissue is cardiac

10   tissue, cerebrovascular tissue, or cerebral tissue.

This invention also provides a method of inhibiting proliferation of fibroblasts or inflammatory cells in a tissue and thereby inhibiting collagen formation comprising

15   contacting the tissue with an inhibitor of VDUP-1 expression. In one embodiment the inhibitor of VDUP-1 expression is a catalytic nucleic acid, a monoclonal antibody, an antisense oligonucleotide, a small molecule, or an RNAi.

20

In one embodiment the subject of any of the above methods is a mammal and in a preferred further embodiment the mammal is a human being. In one embodiment the subject has a cardiovascular disease. In further embodiments the

25   subject has congestive heart failure, has suffered a myocardial infarct, has suffered myocardial ischemia, has angina, or has a cardiomyopathy.

This invention will be better understood by reference to

30   the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow

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thereafter.

EXPERIMENTAL RESULTS (I)

CXC Chemokines Regulate Endothelial Progenitor Cell Migration To The Heart.

5   We investigated the in vivo role of CXC receptor-ligand interactions in mediating chemotaxis of human Endothelial progenitor cell to ischemic tissue and subsequent induction of vasculogenesis, using myocardial infarction in the LAD-ligated nude rat model. As shown in Figure 1a,

10   DiI-labelled human CD34+ cells obtained by G-CSF mobilization (>98% CD34 purity, containing 6-12% CD117^{bright} endothelial progenitor cells) were selectively detected in infarcted myocardium after intravenous injection, but not in myocardium from sham-operated rats. Co-administration of

15   blocking mAbs against rat Cinc (the rat homologue of human IL-8 and Gro-alpha), or against the human surface receptors for these pro-angiogenic chemokines, CXCR1 or CXCR2, reduced myocardial trafficking of human bone marrow-derived CD34+ cells at 48 hours by 40-60% relative to control

20   antibodies (p<0.01), Figure 1b. By two weeks, rats receiving human CD34+ cells demonstrated significantly increased infarct bed microvascularity in comparison to rats receiving saline, Figure 1c, and this was reduced by 50% when anti-CXCR1/2 mAbs were co-administered. Since we

25   have previously shown that the vasculogenic properties of CD34+ cells are abolished after depletion of the minor CD117^{bright} angioblast fraction (7), these results indicate that by regulating angioblast migration to ischemic tissues CXC chemokines influence the development of vasculogenesis

30   at these sites. In contrast, although direct intracardiac injection of IL-8 or SDF-1 at 1.0 μg/ml into non-infarcted hearts resulted in 2.3 and 2.5-fold increases in myocardial infiltration by human CD34+ cells at 48 hours (both p<0.01), Figure 1d, no vasculogenesis was observed at two

weeks under these conditions. Together, these results indicate that following chemokine-induced migration to the infarct zone, differentiation of endothelial progenitor cell to mature endothelial cells and induction of
5   vasculogenesis require additional factors, as yet undefined, which are produced under ischemic conditions.

Inhibiting CXCR4/SDF-1 Interactions Redirects Endothelial Progenitor Cells to The Heart.

10 Although LAD coronary artery ligation resulted in trafficking of intravenously injected human endothelial progenitor cells to the site of ischemic myocardium, it was also accompanied by increased distribution of human cells to rat bone marrow. As shown in Figure 2a, at 2-14 days
15 after intravenous injection of 2 x 10^6 human CD34+ cells bone marrow from LAD-ligated rats contained 5-8 fold higher levels of human CD117^{bright} endothelial progenitor cells compared with bone marrow from normal rats, $p<0.001$. This was presumably due to the proliferative effects of factors
20 in ischemic serum since we have previously shown that culture for 2 days with ischemic serum increases proliferation of CD34+CD117^{bright} human endothelial progenitor cells by 4-5 fold (7). Because the migration of actively cycling CD34+ cells to bone marrow is promoted by SDF-1
25 produced constitutively by marrow stromal cells (31), we investigated whether the distribution of human CD34+CD117^{bright} endothelial progenitor cells to ischemic rat bone marrow involved SDF-1/CXCR4 interactions. As shown in Figure 2b, co-administration of mAbs against either human
30 CXCR4 or rat SDF-1 significantly inhibited migration of intravenously administered human endothelial progenitor cells to ischemic rat bone marrow compared with anti-CD34 control antibody (both $p<0.001$). Moreover, co-administration of mAbs against either human CXCR4 or rat

SDF-1 increased trafficking of CD34+ human endothelial progenitor cells to ischemic rat myocardium by means of 24% and 17%, respectively (both p<0.001), Figure 2c.

5   Capillary Lumen Size Is Dependent On Absolute Angioblast Numbers.

We next examined the relationship between angioblast number, myocardial neovascularization, and protection against myocyte apoptosis. Two days following LAD ligation
10   animals were intravenously injected with G-CSF mobilized CD34+ human cells reconstituted with varying proportions of CD117^{bright} endothelial progenitor cells (10^3, 10^5, 10^5 plus anti-CXCR4 mAb, 2×10^5, and 2×10^5 plus anti-CXCR4 mAb). Similar numbers of DiI-labelled human cells were detected
15   in the infarct zone 48 hours after injecting each cellular population, data not shown. Induction of neovascularization at two weeks was measured by performing quantitative analysis of medium- and large-sized capillaries, defined, respectively, as having 3-6 or >6 contiguous endothelial
20   lining cells. Medium-sized capillaries had mean lumen diameter of 0.020mm + 0.002, while large-sized capillaries had mean lumen diameter of 0.053mm + 0.004 (p<0.001). Notably, large-lumen capillaries overlapped in size with arterioles which could be distinguished by a thin layer
25   containing 2-3 smooth muscle cells of rat origin, as determined by positive staining with desmin and rat MHC class I mAbs. As shown in Figures 3a-c, both the group receiving 2×10^5 endothelial progenitor cells and the one receiving 10^5 endothelial progenitor cells plus anti-CXCR4
30   mAb demonstrated 1.7-fold higher numbers of medium-sized capillaries compared with the other two groups (p<0.01). The group receiving 2×10^5 endothelial progenitor cells additionally demonstrated 3.3-fold higher numbers of large-lumen capillaries compared with the groups receiving

10^3 or 10^5 endothelial progenitor cells (p<0.01), and 2-fold higher numbers of large-lumen capillaries compared with the group receiving 10^5 endothelial progenitor cells plus anti-CXCR4 mAb (p<0.01). As shown in Figure 3d,

5   co-administration of anti-CXCR4 mAb together with the highest concentration of endothelial progenitor cells, 2×10^5, resulted in a further 23% increase in growth of large-lumen capillaries. More strikingly, there was a further 2-fold increase in capillary numbers when 2×10^5

10 endothelial progenitor cells were injected intravenously after direct intracardiac delivery of 1.0 μg/ml SDF-1 into infarcted hearts (p<0.01). Since no similar increase in peri-infarct capillary numbers was seen after intracardiac delivery of IL-8, we interpret these results to indicate

15 that the endogenous IL-8 concentrations in ischemic rat hearts were sufficient to saturate angioblast CXCR1/2 receptors in vivo, whereas intracardiac injection of SDF-1 resulted in a shift in the balance of SDF-1 expression between bone marrow and heart, and consequently resulted in

20 redirected angioblast trafficking to the ischemic heart.

As shown in Figure 3e, the number of apoptotic cardiomyocytes at the infarct rim was significantly reduced in both rats receiving 10^5 endothelial progenitor cells plus

25 anti-CXCR4 mAb and those receiving 2×10^5 endothelial progenitor cells compared with the groups receiving either 10^3 or 10^5 endothelial progenitor cells alone (both p<0.001). Moreover, co-administration of anti-CXCR4 mAb or intracardiac injection of SDF-1 resulted in further

30 reductions in cardiomyocyte apoptosis of 65% and 76%, respectively, Figure 3f (both p<0.001). Together, these data indicate that post-infarct cardioprotection against myocyte apoptosis is dependent on myocardial neovascularization induced by a critical number of

intravenously injected endothelial progenitor cells. This threshold can apparently be lowered by strategies that prevent endothelial progenitor cell redistribution to the bone marrow, such as interrupting CXCR4/SDF-1 interactions,
5   or enhanced SDF-1 expression in the ischemic myocardium.

Capillary Lumen Size As Determinant Of Improvement In Cardiac Function.

We next examined the effect of increasing the number of
10 human endothelial progenitor cells trafficking to ischemic myocardium on long-term myocardial function, defined as the degree of improvement in left ventricular ejection fraction (LVEF) and reduction in left ventricular end-systolic area (LVAs) at 15 weeks after intravenous injection, Figures 4a
15 and b. No improvement in these parameters was observed in the groups receiving 10^3 or 10^5 endothelial progenitor cells in comparison to rats receiving saline alone. In contrast, rats receiving 10^5 endothelial progenitor cells plus anti-CXCR4 mAb demonstrated significant improvement in
20 these parameters, 22 + 2% mean recovery in LVEF and 24 + 4% mean reduction in LVAs (both p<0.001). Even more strikingly, the group receiving 2×10^5 endothelial progenitor cells had a mean recovery in LVEF of 34 + 4% and a mean reduction in LVAs of 37 + 6% (both p<0.001), or 50% further
25 improvement in both parameters. These results were very surprising to us since both groups of animals had demonstrated the same degree of neovascularization involving medium-sized capillaries at two weeks together with similar levels of protection against early apoptosis
30 of cardiomyocytes. This suggested that the additional functional long-term improvement in rats receiving 2×10^5 human endothelial progenitor cells was related to the early development of large-sized capillaries and was mediated through a different mechanism than protection against

myocyte apoptosis.

Large Capillaries Induce Sustained Regeneration Of Endogenous Myocytes.

5   Although myocyte hypertrophy and increase in nuclear ploidy have generally been considered the primary mammalian cardiac responses to ischemia, damage, and overload (1,2) recent observations have suggested that human cardiomyocytes have the capacity to proliferate and

10   regenerate in response to injury (18,19). Therefore, we investigated whether the additive improvement in cardiac function observed after injection of 2×10^5 human endothelial progenitor cells involved induction of cardiomyocyte proliferation and/or regeneration. At two weeks after LAD

15   ligation rats receiving 2×10^5 human endothelial progenitor cells demonstrated numerous "fingers" of cardiomyocytes of rat origin, as determined by expression of rat MHC class I molecules, extending from the peri-infarct region into the infarct zone. Similar extensions were seen less frequently

20   in animals receiving 10^3 and 10^5 endothelial progenitor cells, and very rarely in those receiving saline. As shown in Figure 4c, the islands of cardiomyocytes at the peri-infarct rim in animals receiving 2×10^5 human endothelial progenitor cells contained a high frequency of

25   rat myocytes with DNA activity, as determined by dual staining with mAbs reactive against cardiomyocyte-specific troponin I and rat Ki-67. In contrast, in animals receiving saline there was a high frequency of cells with fibroblast morphology and reactivity with rat Ki-67, but not troponin

30   I, within the infarct zone. The number of cardiomyocytes progressing through cell cycle at the peri-infarct region of rats receiving 2×10^5 human endothelial progenitor cells was 40-fold higher than that at sites distal to the infarct, where myocyte DNA activity was no different than

in sham-operated rats. As shown in Figure 4d, animals receiving 2×10^5 human endothelial progenitor cells had a 20-fold higher number of cell-cycling cardiomyocytes at the peri-infarct rim than that found in non-infarcted hearts

5   (1.19 + 0.2% vs 0.06 + 0.03%, p<0.01) and 3.5-fold higher than in the same region in LAD-ligated controls receiving saline (1.19 + 0.2% vs 0.344 + 0.1%, p<0.01). When 2×10^5 human endothelial progenitor cells were intravenously injected after direct intracardiac delivery of 1.0 μg/ml

10   SDF-1 into infarcted hearts, the number of cell-cycling cardiomyocytes at the peri-infarct rim was increased by a further 1.9-fold compared with intravenous injection of 2×10^5 human endothelial progenitor cells alone (Figure 4e, p<0.01). Thus, intracardiac injection of SDF-1 in

15   combination with intravenous injection of 2×10^5 human endothelial progenitor cells resulted in approximately an 8-fold cumulative increase in cell-cycling cardiomyocytes at two weeks compared with LAD-ligated controls receiving saline, and translated into over 4-fold greater LVEF

20   improvement, determined by echocardiography, compared with intravenous injection of 2×10^5 endothelial progenitor cells alone (Figure 4f, p<0.01). Co-administration of anti-CXCR4 mAb augmented LVEF improvement by 2.8-fold (p<0.01) while intracardiac injection of IL-8 conferred no additive

25   benefit.

Quantitation of the ratio of fibrous tissue to myocytes at 15 weeks demonstrated significantly reduced proportions of scar/normal left ventricular myocardium in both the group

30   receiving 2×10^5 endothelial progenitor cells and the one receiving 10^5 endothelial progenitor cells plus anti-CXCR4 mAb, respectively 13% and 21% compared with 37-46% for each of the other groups (p<0.01), Figure 4g. Since both groups had nearly identical levels of protection against early

cardiomyocyte apoptosis, we infer that the additional 38% reduction in scar/myocyte ratio seen in the group injected with 2×10^5 endothelial progenitor cells actually reflects proliferation/regeneration of endogenous rat cardiomyocytes

5   induced by nutrient supply from large-vessel neovascularization. This presumably accounts for the increase in functional improvement seen in this group. We conclude that the ratio of scar size to left ventricular muscle mass reflects, in part, positive effects imparted by

10 the ability of the residual myocardium to proliferate and regenerate, in addition to negative effects of the initial infarct size and positive effects of anti-apoptotic, cardioprotective mechanisms. The overall effects of medium- and large-size neovasculature combining to both protect

15 against myocyte apoptosis and induce myocyte proliferation/regeneration are shown dramatically in Figure 4h where, in contrast to saline controls, injection with 2×10^5 endothelial progenitor cells resulted in almost complete salvage of the anterior myocardium, normal septal

20 size and minimal collagen deposition.

Effecting Regeneration

PAI-1-inhibiting catalytic nucleic acid augments human

25 angioblast-dependent cardiomyocyte regeneration.

We investigated whether possible neovascularization induced by a catalytic nucleic acid (designated E2) capable of inhibiting expression of PAI-1 was associated with

30 cardiomyocyte regeneration. Injection of E2 alone did not induce cardiomyocyte regeneration despite the increase in neovascularization. Combining E2 injection with intravenously delivered human endothelial progenitor cells

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strikingly increased the degree of cardiomyocyte regeneration, to levels 7.5-fold higher than in saline controls (p<0.01). A scrambled DNA control enzyme (E0) had no such effect. Moreover, whereas E2 alone did not improve

5   myocardial function, as determined by recovery in left ventricular ejection fraction at two weeks combining E2 with human endothelial progenitor cells resulted in almost doubling of the positive effect of endothelial progenitor cells alone on cardiac functional recovery. These results

10 emphasize the importance of cardiomyocyte regeneration as the primary mechanism by which cardiac function is improved after infarction. Since combining E2 with human endothelial progenitor cells resulted in 62% greater numbers of large capillaries at the peri-infarct rim than use of either

15 approach alone, these results indicate that angioblast-induced cardiomyocyte regeneration and improvement in cardiac function can be optimized by use of synergistic approaches, such as strategies that inhibit PAI-1 expression, which augment neovascularization either

20 directly or through angioblast-dependent processes.

We further discovered that intravenous injection of human CD34+CD117bright angioblasts resulted in a four-fold increase in proliferation/regeneration of rat

25 cardiomyocytes at the peri-infarct region relative to saline controls, as determined by dual staining for troponin and Ki67 (p<0.01). Combining E2 injection with intravenously delivered human angioblasts strikingly increased the degree of cardiomyocyte regeneration, to

30 levels 7.5-fold higher than in saline controls (p<0.01). The scrambled DNA enzyme E0 had no such effect.

Gene effects on myocyte proliferation

We hypothesized that myocardial neovascularization induces the signals required to elicit myocyte proliferation, and therapeutic intervention mimicking this could have striking implications for repair and regeneration of hearts damaged

5   by episodes of ischemia or other insults.

To begin approaching this complex problem, we have employed the technique of cDNA Subtractive Hybridization. This technique enables comparison of the pattern of gene

10   expression between two different sets of conditions. Our initial approach was to compare which genes are differentially expressed between hearts from normal rats and rats who have undergone left anterior descending (LAD) coronary artery ligation 48 hours earlier. We hypothesized

15   that whatever the altered pattern of gene over- or underexpression after 48 hours of ischemia, neovascularization would result in a reversal in the pattern towards that seen in the non-ischemic rat heart.

20   Using cdNA subtractive hybridization we observed a striking reciprocal change in expression of a group of genes whose function is linked through their regulation of cellular apoptosis and cell cycle progression following oxidative stress and other inducers of DNA damage. Whereas expression

25   of certain antioxidant genes such as superoxide dismutase was upregulated in ischemic tissue, the antioxidant stress responsive genes induced by hemin, notably heme binding protein 23 (HBP23) and glutathione-S-transferase, were downregulated. Moreover, a recently-identified protein,

30   Vitamin D3 Up-Regulated Protein1 (VDUP1), whose mRNA expression is induced by hydrogen peroxide (H_2O_2) following oxidative stress and whose function counterbalances that of HBP23, was upregulated in ischemic hearts. These findings

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are particularly striking when considered in the context of previous observations that the presence of deficits in antioxidants and increased oxidative stress accompanying myocardial infarction appear to be directly implicated in

5   the pathogenesis of post-infarct heart failure (74,75).

As shown in Figure 5, using RT-PCR, the reciprocal changes in mRNA expression of HBP23 and VDUP1 in ischemic vs normal rat hearts were confirmed. Moreover, mRNA expression of

10   these two genes returned to normal in rat tissue two weeks after intravenous injection of human adult bone marrow-derived progenitors and infarct zone neovascularization. In contrast, in LAD-ligated rat hearts receiving saline, no change in mRNA expression of these

15   genes was observed at two weeks in comparison to the pattern observed at 48 hours. To comprehend the relationship between the effects of neovascularization on these altered expression patterns of HBP23 and VDUP1 following ischemia, and the observed protection against

20   cardiomyocyte apoptosis together with induction of cardiomyocyte proliferation/regeneration, it is important to understand in detail the molecular effects of the products encoded by these genes on cellular apoptosis and cell cycle progression.

25

When cells proliferate, the mitotic cycle progression is tightly regulated by an intricate network of positive and negative signals. Progress from one phase of the cell cycle to the next is controlled by the transduction of mitogenic

30   signals to cyclically expressed proteins known as cyclins and subsequent activation or inactivation of several members of a conserved family of serine/threonine protein kinases known as the cyclin-dependent kinases (cdks) (67). Growth

arrest observed with such diverse processes as DNA damage, terminal differentiation, and replicative senescence is due to negative regulation of cell cycle progression by two functionally distinct families of Cdk inhibitors, the Ink4

5   and Cip/Kip families (64). The cell cycle inhibitory activity of p21Cip1/WAF1 is intimately correlated with its nuclear localization and participation in quaternary complexes of cell cycle regulators by binding to G1 cyclin-CDK through its N-terminal domain and to

10   proliferating cell nuclear antigen (PCNA) through its C-terminal domain (68-71). The latter interaction blocks the ability of PCNA to activate DNA polymerase, the principal replicative DNA polymerase (72). For a growth-arrested cell to subsequently enter an apoptotic

15   pathway requires signals provided by specific apoptotic stimuli in concert with cell-cycle regulators. For example, caspase-mediated cleavage of p21, together with upregulation of cyclin A-associated cdk2 activity, have been shown to be critical steps for induction of cellular apoptosis by either

20   deprivation of growth factors (73) or hypoxia of cardiomyocytes (74).

The apoptosis signal-regulating kinase 1 (ASK1) is a pivotal component in the mechanism of cytokine- and stress-induced

25   apoptosis (75,76). Under basal conditions, resistance to ASK1-mediated apoptosis appears to be the result of complex formation between ASK1, cytoplasmic p21Cip1/WAF1 (77), and the thiol reductase thioredoxin (TRX) (78). Intact cytoplasmic expression of p21Cip1/WAF1 appears to be

30   important for both prevention of apoptosis in response to ASK1 (75) and in maintaining a state of terminal differentiation (77). Moreover, the reduced form of TRX, but not the oxidized form, binds to the N-terminal portion of ASK1 and is a physiologic inhibitor of ASK1-mediated

51

cellular apoptosis (78). The recently-identified protein VDUP1 has been shown to compete with ASK1 for binding of the reduced form of TRX (78,79), resulting in augmention of ASK1-mediated apoptosis (80). This indicates that

5 ASK1-mediated cellular apoptosis is increased by processes that result in a net dissociation of TRX from ASK1, such as either generation of TRX-VDUP1 complexes or generation of oxidised TRX by changes in cellular redox status accompanying oxidative stress.

10

TRX and glutathione constitute the major cellular reducing systems that maintain the thiol-disulfide status of the cytosol (81). The redox-active/dithiol active site of TRX is highly conserved across all species,

15 Trp-Cys-Gly-Pro-Cys-Lys. The two cysteine residues at the active site, Cys-32 and Cys-35, undergo reversible oxidation-reduction reactions catalyzed by a NADPH-dependent enzyme TRX reductase. These reactions involve electron transfer via disulfide bridges formed with members of a

20 family of antioxidant enzymes known as peroxiredoxins (Prxs), which show peroxidase activity (82,83). Prxs are distinct from other peroxidases in that they have no cofactors, such as metals or prosthetic groups. Prxs generally have two conserved cysteines at the N- and

25 C-terminal regions (84), and their antioxidant effects are coupled with the physiological electron donor activity of the TRX system (82,85, 86). Prxs with 95-97% sequence homology have been identified in rats (Heme-binding protein 23, HBP23) (87), mice (mouse macrophage stress protein 23,

30 MSP23) (88) and humans (proliferation-associated gene product, PAG (89) and human natural killer cell-enhancing factor A (90)).

Prxs are members of a repertoire of oxidative stress responsive genes whose expression is regulated by NF-E2-related factor 2 (Nrf2) which binds to an anti-oxidant responsive element (ARE) present in the promoter of each
5   (91). These include glutathione-S-transferase, heme oxygenase-1, and TRX. Under basal conditions, Nrf2 is bound to a specific protein, Keap1, in the cytosol (92). However, under conditions of oxidative stress Nrf2 dissociates from Keap1 and translocates to the nucleus where it induces
10 transcriptional activation of the anti-oxidant genes containing ARE motifs. Although the precise extracellular signalling pathways have not been elucidated, nuclear translocation of Nrf2 and subsequent ARE activation appear to be dependent on pathways activated by
15 phosphatidylinositol 3-kinase (PI3 kinase) (93). In addition, hemin is a potent inducer of Nrf2 dissociation from Keap1, resulting in TRX gene transcription through the ARE (94).

20 During periods of rapid changes in cellular redox Prxs presumably serve to maintain the cytosolic levels of reduced TRX by accepting electrons from the oxidized form of TRX. This homeostatic mechanism likely enables maintenance of sufficient levels of reduced TRX to ensure adequate binding
25 to ASK1 and prevention of cellular apoptosis. If the endogenous Prx system is overloaded, as might occur during changes in cellular redox when excess oxidized TRX is generated, cellular apoptosis will occur through the unopposed effects of ASK1. To counteract this,
30 transcriptional activation of Prxs must occur following oxidative stress via nuclear translocation of Nrf2. This can be achieved either by Nrf2 dissociation from Keap1 via hemin- and PI3 kinase-dependent mechanisms (93,94), or by increasing Nrf2 mRNA and protein expression as occurs

following increase in oxygen tension (95,96).

In addition to directly interacting with TRX, the Prx gene products (PAG, HBP23, MSP23, NKEF, etc) specifically bind

5   the SH3 domain of c-Abl, a non-receptor tyrosine kinase, inhibiting its activation by various stimuli, including agents that damage DNA (97). c-Abl activation through the SH3 domain induces either arrest of the cell cycle in phase G1 or cellular apoptosis (98). Cell cycle arrest is

10   dependent on the kinase activity of c-Abl (96) and is mediated by the ability of c-Abl to downregulate the activity of the cyclin-dependent kinase Cdk2 and induce the expression of p21 (99). The apoptotic effects of c-Abl are dependent on the ability of nuclear c-Abl to phosphorylate

15   p73, a member of the p53 family of tumor-suppressor proteins which can induce apoptosis (100,101). Recently, it has been shown that cytoplasmic, rather than nuclear, forms of c-Abl are activated by H_2O_2 and that this results in mitochondrial localization of c-Abl, c-Abl dependent cytochrome c release,

20   and cellular apoptosis following oxidative stress (102,103). By associating with c-Abl in vivo, the PAG gene product (and presumably the other Prxs) can inhibit tyrosine phosphorylation induced by c-Abl overexpression and rescue cells from both the cytostatic and pro-apoptotic effects of

25   the activated c-Abl gene product (97).

Our finding that Nrf2-dependent oxidative stress responsive genes are downregulated following myocardial ischemia likely reflects direct effects of hemin and oxygen deprivation.

30   The end result of Prx downregulation in the ischemic heart would be augmentation in ASK1-dependent cellular apoptosis as well as Abl-dependent apoptosis and cell cycle arrest. The observed parallel increase in VDUP1 expression would

further augment ASK1-dependent cellular apoptosis. Thus, the ratio in expression of PAG or other Prx mRNA or protein to VDUP1 mRNA or protein can form the basis of a diagnostic assay to predict the degree of risk for cardiomyocyte

5   apoptosis and cell cycle arrest after ischemia, as well as enable monitoring of the response to specific therapy after myocardial ischemia that protects cardiomyocytes against apototic   death   and   enhances   myocardial proliferation/regeneration.

10

Reversing the reduced expression of the Prxs following myocardial ischemia would increase Prxs in the heart in order to protect the ischemic myocardium against apoptosis through both c-Abl inhibition and reduction of oxidised TRX,

15   and to enable cardiomyocyte proliferation/regeneration by inhibiting the effects of c-Abl on cell cycle progression from G1 to S phase.

Increasing Nrf2 mRNA or causing dissociation of Nrf2 protein

20   from   Keap1,   or   preferably   cause   both   to   occur simultaneously, in the setting of myocardial ischemia in order to increase transcription and activity of members of a repertoire of oxidative stress responsive genes whose expression is regulated by binding of Nrf2 to an

25   anti-oxidant responsive element (ARE) in their promoters, including the Prxs, TRX and glutathione-S-transferase would result in both protection of cardiomyocytes against apoptosis as well as induce cardiomyocyte cell cycle progression following oxidative stress.

30

Reducing the expression of VDUP1 following myocardial ischemia would protect the ischemic myocardium against apoptosis by reducing binding of TRX to VDUP1, and

consequently increasing TRX-ASK1 interactions.

Neovascularization of the myocardium, by either bone
marrow-derived endothelial progenitors or any other process,
5   is an example of one method which causes induction of Prx
expression and reduction in VDUP1 expression after
myocardial ischemia, and results in both protection against
redox-mediated apoptosis and induction of myocardial
proliferation/regeneration.

10

Small molecules which specifically inhibit binding of Nrf2
to Keap1 would be expected to have similar protective
effects against cardiomyocyte apoptosis and to induce
myocardial proliferation/regeneration after ischemia.
15   Similarly, small molecules that specifically inhibit binding
of TRX to VDUP1, would be expected to have similar
protective effects against cardiomyocyte apoptosis after
ischemia.

20   Use of small molecules to specifically inhibit c-Abl
tyrosine kinase activation after myocardial ischemia would
be expected to have similar protective effects against
cardiomyocyte apoptosis and to induce myocardial
proliferation/regeneration after ischemia. A specific
25   example of a small molecule to inhibit c-Abl tyrosine kinase
activation is STI-571. Use of this or related molecules
after myocardial infarction would protect against
cardiomyocyte apoptosis and induce myocardial
proliferation/regeneration.

30

DNA Enzyme Specific For VDUP-1 Cleaves Synthetic Rat VDUP-1
Oligonucleotide

By subtractive hybridization, we found that mRNA expression of the protein VDUP1 is significantly increased in the heart after acute ischemia. VDUP1 has been shown to bind the cytosolic protein thioredoxin, TRX, which functions to

5   maintain the thiol-disulfide status of the cytosol. By binding to reduced forms of TRX, VDUP1 prevents the ability of reduced TRX to undergo reversible oxidation-reduction reactions catalyzed by a NADPH-dependent enzyme, TRX reductase. This results in cellular apoptosis due to

10   excessive cytosolic and mitochondrial production of oxygen radicals.

VDUP1 competes for TRX binding with another cytosolic protein that is normally bound to reduced TRX, the apoptosis

15   signal-regulating kinase 1 (ASK1). ASK-1 is a pivotal component in the mechanism of cytokine- and stress-induced apoptosis. Its activation results in excessive phosphorylation and activation of p38 MAP kinase, a principal mediator of cellular apoptosis. The reduced form

20   of TRX, but not the oxidised form, binds to the N-terminal portion of ASK1 and is a physiologic inhibitor of ASK1-mediated cellular apoptosis. Binding of VDUP1 to TRX results in a net dissociation of ASK1 from TRX, potentially resulting in augmentation of ASK1-mediated apoptosis via p38

25   MAP kinase-dependent pathways.

With respect to cardiac overexpression of VDUP1, the anticipated effects would be those due to excessive p38 MAP kinase activation and oxidative redox damage, including

30   cardiomyocyte apoptosis, fibroblast proliferation, collagen secretion and scar formation. Similar effects would be expected to be the consequence of VDUP1 overexpression in other tissues undergoing acute or chronic ischemic injury,

for   example   the   brain   following   cerebrovascular
ischemia/stroke.

5   We have developed a DNA enzyme targeting the VDUP1 mRNA. The
       DNA enzyme, once delivered to the ischemic myocardium (or
       other ischemic tissues such as the brain) can inhibit local
       VDUP1 mRNA and protein expression, thus reducing p38 MAP
       kinase activation and oxidative damage.

10   As shown in figure 12, at enzyme concentrations ranging from
       0.05uM to 5uM the sequence-specific VDUP1 DNA enzyme cleaved
       a synthetic rat VDUP1 oligonucleotide in a concentration-
       and time-dependent manner.

15   VDUP-1 DNAzyme Reduces Fibroblast Proliferation And Protects
       Against Cardiomyocyte Apoptosis.   As shown in figure 13(a),
       intramyocardial injection of the rat sequence-specific VDUP1
       DNA enzyme at 48 hours after LAD ligation resulted in a 75%
       mean inhibition of proliferating cardiac fibroblasts in the
20   infarct zone two weeks later in comparison to injection of
       scrambled DNA enzyme control (p<0.01).   In addition, as seen
       in figure 13(b), injection of VDUP1 DNA enzyme resulted in
       20%   mean   reduction   in   apoptotic   cardiomyocytes   at   the
       peri-infarct region relative to injection with the scrambled
25   DNA enzyme control (p<0.05).

       VDUP-1 DNAzyme Reduces Myocardial Scar And Improves Cardiac
       Function After Acute Infarction.   Inhibition of fibroblast
       proliferation   and   cardiomyocyte   apoptosis   resulted   in
30   significant   reduction   of   mature   scar   deposition   in   the
       infarct zone, from a mean of 35% for animals receiving
       control scrambled DNA enzyme to a mean of 20% for those

receiving VDUP1 DNA enzyme, figure 14(a) (p<0.01). Most dramatic was the effect on cardiac function. As shown in figure 14(b), animals receiving VDUP1 DNA enzyme demonstrated a 50% mean recovery in cardiac function, as

5   determined by ejection fraction, whereas no improvement was seen in animals receiving scrambled control DNA enzyme (p<0.01).

Clearly, VDUP1 DNA enzyme prevents cardiomyocyte apoptosis,

10   cardiac fibroblast proliferation and scar formation, resulting in significant improvement in cardiac function after acute ischemia. These effects are presumably due to prevention of p38 MAP kinase activation and protection against redox damage. Similar results might be obtained by

15   administering the VDUP1 DNA enzyme to other tissue sites of reduced blood flow such as the brain after cerebrovascular ischemia.

G-CSF

20   G-CSF is a more potent inducer of neovascularization after myocardial infarction than GM-CSF. As shown in figure 15(a), rats injected subcutaneously with human G-CSF at 10 ug/kg for four days starting at two days after myocardial infarction induced by left anterior descending (LAD)

25   coronary artery ligation demonstrated approximately 7.5-fold greater numbers of large-diameter blood vessels at the peri-infarct region two weeks later relative to saline-treated controls (p<0.01). Rat GM-CSF administered at the same dosage regimen was less effective, though still

30   inducing 4-fold greater numbers of large-lumen vessels than in control animals.

Additionally, G-CSF is a more potent inhibitor of cardiomyocyte apoptosis after myocardial infarction than GM-CSF. G-CSF injection was a more potent agent for protecting against cardiomyocyte apoptosis than GM-CSF used at the same

5   dosage regimen, figure 15(b). G-CSF administration resulted in 36 + 16% reduction in the numbers of apoptotic cardiomyocytes at the peri-infarct region by two weeks relative to saline-treated controls (p<0.01), whereas GM-CSF only reduced apoptotic cardiomyocyte numbers by 12 + 9%.

10

G-CSF is a more potent inducer of cardiomyocyte regeneration and functional cardiac recovery after myocardial infarction than GM-CSF. Next we examined the effects of bone marrow mobilization on cardiomyocyte cell cycling/regeneration and

15   on functional cardiac recovery. As shown in figure 16(a), rats injected subcutaneously with human G-CSF at 10 ug/kg for four days starting at two days after myocardial infarction demonstrated 3.2-fold greater numbers of cycling cardiomyocytes two weeks later at the peri-infarct region

20   relative to saline-treated controls (p<0.05). Rat GM-CSF administered at the same dosage regimen was less effective, resulting in 2.6-fold greater numbers of cycling cardiomyocytes. As shown in figure 16(b), this correlated with functional cardiac recovery. Whereas saline-treated

25   animals had a 17% mean loss in cardiac function from day 2 to day 14 after infarction as measured by ejection fraction, GM-CSF treated animals had a loss of only 10% in cardiac function, and G-CSF treated animals actually had a 10% mean improvement in cardiac function (P<0.01). We interpret these

30   functional data to reflect the superior effects of G-CSF bone marrow mobilization on myocardial neovascularization, protection against cardiomyocyte apoptosis and induction of cardiomyocyte cell cycling.

Anti-CXCR4 Antibodies

Intravenous administration of anti-CXCR4 antibody increases myocardial neovascularization and improves cardiac function after acute infarction. The major mechanism by which G-CSF

5   causes mobilization of bone marrow elements is through interruption of interactions between the chemokine receptor CXCR4 on bone marrow resident stem cells and its ligand SDF-1. G-CSF induces both cleavage of the N-terminus of CXCR4 and accumulation of serine proteases which directly

10   cleave and inactivate SDF-1. To examine whether similar mechanisms were responsible for the effects of G-CSF administration on myocardial neovascularization and improvement in cardiac function, we investigated the effect of interrupting CXCR4-SDF1 interactions by intravenously

15   administering a monoclonal anti-CXCR4 antibody 48 hours after LAD ligation. As shown in figure 17(a) at two weeks after antibody administration animals receiving anti-CXCR4 mAb demonstrated a two-fold increase in neovascularization at the peri-infarct region compared with control animals

20   receiving either saline or anti-CXCR2 mAb. Moreover, as seen in figure 17(b) anti-CXCR4 treated animals demonstrated a mean recovery in ejection fraction of 10% at two weeks whereas those receiving anti-CXCR2 mAb had a mean loss in cardiac function of 8% (p<0.05). These data support the

25   concept that the observed effects of G-CSF administration result from interrupting CXCR4 interactions in the bone marrow, enabling endothelial progenitor cells to be mobilized to the peripheral circulation, and to home to ischemic myocardium where the resultant neovascularization

30   results in improvement in cardiac function.

SDF-1

SDF-1 mRNA expression is not induced early in acutely

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ischemic myocardium, and its late induction is inhibited by
GM-CSF. Next, we sought to identify a strategy by which the
effects of GM-CSF could be augmented to approach those seen
with G-CSF treatment alone, such as increasing chemotactic
5   signals in the acutely ischemic myocardium. Since
chemotaxis of CD34+ bone marrow stem cells is regulated by
interactions between CXCR4 receptors on the CD34+ cells and
the CXC chemokine SDF-1, we investigated whether SDF-1 mRNA
expression was induced in the acutely ischemic myocardium.
10   As shown in figure 18, no difference in myocardial SDF-1
mRNA was observed at 48 hours after LAD ligation in
experimental animals relative to non-ischemic controls. By
two weeks after infarction, myocardial SDF-1 mRNA expression
had increased by 3.3-fold compared with saline-treated
15   controls (p<0.01). We interpreted this delayed production
of SDF-1 as most likely reflecting elaboration by
infiltrating cells such as macrophages. In contrast,
systemic GM-CSF administration was accompanied by
approximately 4.5-fold inhibition in SDF-1 mRNA expression
20   at two weeks, to levels actually lower than in non-ischemic
controls. Since SDF-1 is a potent chemotactic factor for
endothelial progenitor cells, these data suggested that
systemic GM-CSF administration may result in suboptimal
myocardial homing of endothelial progenitors due to
25   decreased myocardial expression of chemotactic ligands such
as SDF-1.

Intramyocardial injection of SDF-1 after acute myocardial
infarction results in neovascularization and protection
30   against cardiomyocyte apoptosis. To determine whether
altered SDF-1 expression in the acutely ischemic heart
affects endothelial progenitor cell chemotaxis and
cardiomyocyte function, we examined the effects of direct
intramyocardial injection of SDF-1 protein at 48 hours after

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LAD ligation. As shown in figures 19 (a) and (b),
intramyocardial injection of 4ug/kg human recombinant SDF-1
in a total volume of 0.2 ml at 5 peri-infarct sites two days
post-LAD ligation resulted by two weeks in a 5-fold increase
5   in neovascularization and a 44 + 9% reduction in apoptotic
cardiomyocytes at the peri-infarct region relative to
control animals receiving intramyocardial saline injections
(both p<0.05). The effects of intramyocardial SDF-1
injection on neovascularization and on extent of protection
10   against cardiomyocyte apoptosis were in strikingly close
parallel   to   the   results   obtained   with   systemic
administration   of   G-CSF.   Although   addition   of
subcutaneously administered GM-CSF resulted in a synergistic
increase   in   myocardial   neovascularization,   no   further
15   benefit in protection against cardiomyocyte apoptosis was
observed. These results suggest that there is a finite
amount of protection against cardiomyocyte apoptosis that
can be induced by neovascularization, and that this cannot
be improved upon by further by induction of additional new
20   blood vessels.

Intramyocardial injection of SDF-1 induces cardiomyocyte
regeneration. As shown in figure 20(a), intramyocardial
injection of 4ug/kg human recombinant SDF-1 in a total
25   volume of 0.2 ml at 5 peri-infarct sites two days post-LAD
ligation resulted in a 4.5-fold increase in the number of
cycling cardiomyocytes at the peri-infarct region relative
to saline-injected controls (p<0.01). Addition of systemic
GM-CSF administration resulted in synergistic effects on
30   cardiomyocyte regeneration. Notably, the numbers of cycling
cardiomyocytes at the peri-infarct region in animals
receiving combined therapy with SDF-1 and GM-CSF
significantly exceeded those seen in animals receiving G-CSF
alone (7-fold versus 3.2-fold above saline treated controls,

p<0.01). Since mean numbers of large-lumen vessels at the peri-infarct region were similar in animals receiving combined therapy with SDF-1 and GM-CSF and in those receiving G-CSF alone (8.0 vs 7.5/high power field), these

5   data suggest that SDF-1 enhanced cardiomyocyte cycling/regeneration by an alternative mechanism in addition to induction of neovascularization.

       Intramyocardial injection of SDF-1 improves cardiac function
10   and is synergistic with bone marrow mobilization. Intramyocardial injection of SDF-1 resulted in a similar degree of functional myocardial recovery as was seen with systemic G-CSF administration (10% mean improvement in ejection fraction between days 2 and 14 post-LAD ligation

15   compared with 10% mean improvement for G-CSF treated animals and 17% mean loss for saline-treated controls, p<0.01 for both treatment arms). Most strikingly, combining SDF-1 injection with bone marrow mobilization by GM-CSF resulted in significant augmentation in functional recovery (21% mean

20   improvement in ejection fraction, p<0.01), figure 20(b). These data indicate that intramyocardial administration of SDF-1 causes improvement in cardiac function after acute ischemia through two separate mechanisms, a direct mechanism which involves induction of cardiomyocyte cycling and

25   regeneration and an indirect mechanism operating through enhanced chemotaxis of mobilized bone marrow-derived endothelial progenitors and cardiac neovascularization. Optimal results are likely to be seen when combining intramyocardial SDF-1 treatment with the most potent

30   mobilizers of bone marrow endothelial progenitors, including G-CSF and other agents that inhibit CXCR4 interactions with SDF-1 in the bone marrow.

METHODS AND MATERIALS

<u>Purification and characterization of cytokine-mobilized human CD34+ cells</u>: Single-donor leukopheresis products were
5   obtained from humans treated with recombinant G-CSF 10 mg/kg (Amgen, CA) sc daily for four days. Donors were healthy individuals undergoing standard institutional procedures of bone marrow mobilization, harvesting and isolation for allogeneic stem cell transplants. Mononuclear cells were
10   separated by Ficoll-Hypaque, and highly-purified CD34+ cells (>98% positive) were obtained using magnetic beads coated with anti-CD34 monoclonal antibody (mAb) (Miltenyi Biotech, CA). Purified CD34 cells were stained with fluorescein-conjugated mAbs against CD34 and CD117 (Becton
15   Dickinson, CA), AC133 (Miltenyi Biotech, CA), CD54 (Immunotech, CA), CD62E (BioSource, MA), VEGFR-2, Tie-2, vWF, eNOS, CXCR1, CXCR2, and CXCR4 (all Santa Cruz Biotech, CA), and analyzed by four-parameter fluorescence using FACScan (Becton Dickinson, CA). Cells positively selected
20   for CD34 expression were also stained with phycoerythrin (PE)-conjugated anti-CD117 mAb (Becton Dickinson, CA), and sorted for bright and dim fluorescence using a Facstar Plus (Becton Dickinson) and a PE filter. Intracellular staining for GATA-2 was performed by permeabilizing one million cells
25   from each of the brightly and dimly fluorescing cell populations using a Pharmingen Cytofix/Cytoperm™ kit, incubating for 30 minutes on ice with 10μl of fluorochrome-conjugated mAbs against both CD117 and CD34 surface antigens (Becton Dickinson, CA). After resuspension
30   in 250μl of Cytofix/Cytoperm™ solution for 20 minutes at 4 degrees C, cells were incubated with a fluorochrome-labeled mAb against GATA-2 (Santa Cruz Biotech, CA) or IgG control for 30 minutes at 4 degrees C, and analyzed by three-parameter flow cytometry.

Chemotaxis of human bone-marrow derived endothelial progenitors: Highly-purified CD34+CD117^{bright} cells (>98% purity) were plated in 48-well chemotaxis chambers fitted with membranes (8 mm pores) (Neuro Probe, MD). After

5   incubation for 2 hours at 37°C, chambers were inverted and cells were cultured for 3 hours in medium containing IL-8, SDF-1 alpha/beta, and SCF at 0.2, 1.0 and 5.0 μg/ml. The membranes were fixed with methanol and stained with Leukostat™ (Fischer Scientific, Ill). Chemotaxis was

10 calculated by counting migrating cells in 10 high-power fields.

Animals, surgical procedures, injection of human cells, and quantitation of cellular migration into tissues: Rowett

15 (rnu/rnu) athymic nude rats (Harlan Sprague Dawley, Indianapolis, Indiana) were used in studies approved by the "Columbia University Institute for Animal Care and Use Committee". After anesthesia, a left thoracotomy was performed, the pericardium was opened, and the left anterior

20 descending (LAD) coronary artery was ligated. Sham-operated rats had a similar surgical procedure without having a suture placed around the coronary artery. For studies on cellular migration, 2.0 x 10^6 CD34+ cells obtained from a single donor after G-CSF mobilization were injected into the

25 tail vein 48 hours after LAD ligation either alone or together with 50 μg/ml monoclonal antibody (mAb) with known functional inhibitory activity against either human CXCR1, human CXCR2, human CXCR4, rat SDF-1 (all R & D Systems, MN), human CD34 (Pharmingen, CA), or rat IL-8

30 (ImmunoLaboratories, Japan). Controls received either isotype control antibodies at the same concentration or saline after LAD ligation. Prior to injection, 2.0 x 10^6 human cells were incubated with 2.5 μg/mL of the fluorescent carbocyanine DiI dye (Molecular Probes) for 5 minutes at

37°C and 15 minutes at 4°C.   After washing in PBS, DiI-labeled human cells were resuspended in saline and injected intravenously. 2.0 x 10^6 CD34+ human cells were also injected into the tail vein of sham-operated or

5 LAD-ligated rats receiving three intramyocardial injections of 1.0 µg/ml recombinant human IL-8, SDF-1, SCF or saline. Each group consisted of 6-10 rats. Quantitation of myocardial infiltration after injection of human cells was performed by assessment of DiI fluorescence in hearts from

10 rats sacrificed 2 days after injection (expressed as number of DiI-positive cells per high power field, minimum 5 fields examined per sample). Quantitation of rat bone marrow infiltration by human cells was performed in 12 rats at baseline, days 2, 7, and 14 by flow cytometric and RT-PCR

15 analysis of the proportion of HLA class I-positive cells relative to the total rat bone marrow population. For studies on neoangiogenesis and effects on myocardial viability and function, 2.0 x 10^6 DiI-labelled human CD34+ cells obtained from a single donor after G-CSF mobilization

20 were reconstituted with 10^3, 10^5, or 2.0 x 10^5 immunopurified CD34+CD117^{bright} cells, and injected into the rat tail vein 48 hours after LAD ligation, in the presence or absence of a mAb with known inhibitory activity against CXCR4. Each group consisted of 6-10 rats. Histologic and functional studies

25 were performed at 2 and 15 weeks.

Measurement of rat CXC chemokine mRNA and protein expression: Poly(A)+ mRNA was extracted by standard methods from the hearts of 3 normal and 12 LAD-ligated rats.

30 RT-PCR was used to quantify myocardial expression of rat IL-8 and Gro-alpha mRNA at baseline and at 6, 12, 24 and 48 hours after LAD ligation after normalizing for total rat RNA as measured by GAPDH expression. After priming with oligo (dT) 15-mer and random hexamers, and reverse transcribed

with Moloney murine lymphotrophic virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA), cDNA was amplified in the polymerase chain reaction (PCR) using Taq polymerase (Invitrogen, Carlsbad, CA, USA), radiolabeled

5   dideoxy-nucleotide ([a32P]-ddATP: 3,000 Ci/mmol, Amersham, Arlington Heights, IL), and primers for rat Cinc (rat homologue of human IL-8/Gro-alpha and GAPDH (Fisher Genosys, CA). Primer pairs (sense/antisense) for rat Cinc and GAPDH were, gaagatagattgcaccgatg (SEQ ID NO:4)/catagcctctcacatttc

10 SEQ ID NO:5), gcgcccgtccgccaatgagctgcgc SEQ ID NO:6)/cttggggacacccttcagcatcttttgg SEQ ID NO:7), and ctctacccacggcaagttcaa SEQ ID NO:8)/gggatgaccttgcccacagc SEQ ID NO:9), respectively. The labelled samples were loaded into 2% agarose gels, separated by electrophoresis, and

15 exposed for radiography for 6 h at -70°C. Serum levels of rat IL-8/Gro-alpha were measured at baseline and at 6, 12, 24 and 48 hours after LAD ligation in four rats by a commercial ELISA using polyclonal antibodies against the rat IL-8/Gro homologue Cinc (ImmunoLaboratories, Japan). The

20 amount of protein in each serum sample was calculated according to a standard curve of optical density (OD) values constructed for known levels of rat IL-8/Gro-alpha protein. Anti-Cinc antibodies were also used according to the manufacturer's instructions at 1:200 dilution in

25 immunohistochemical studies to identify the cellular source of Cinc production in rat myocardium after LAD ligation. Positively-staining cells were visualized as brown through the Avidin/Biotin system described below.

30 <u>Histology and measurement of infarct size</u>:   Following excision at 2 and 15 weeks, left ventricles from each experimental animal were sliced at 10-15 transverse sections from apex to base. Representative sections were fixed in formalin and stained for routine histology (H&E) to

determine cellularity of the myocardium, expressed as cell number per high power field (HPF) (600x). A Masson trichrome stain was performed, which labels collagen blue and myocardium red, to evaluate collagen content on a

5   semiquantitative scale (0-3+), with 1+ light blue, 2+ light blue and patches of dark blue, and 3+ dark blue staining. This enabled measurement of the size of the myocardial scar using a digital image analyzer. The lengths of the infarcted surfaces, involving both epicardial and endocardial regions,

10 were measured with a planimeter digital image analyzer and expressed as a percentage of the total ventricular circumference. Final infarct size was calculated as the average of all slices from each heart. All studies were performed by a blinded pathologist. Infarct size was

15 expressed as percent of total left ventricular area. Final infarct size was calculated as the average of all slices from each heart.

Quantitation of capillary density: In order to quantitate capillary density and species origin of the capillaries,

20 additional sections were stained freshly with mAbs directed against rat or human CD31 (Serotec, UK, and Research Diagnostics, NJ, respectively), factor VIII (Dako, CA), and rat or human MHC class I (Accurate Chemicals, CT). Arterioles were differentiated from large capillaries by the

25 presence of a smooth muscle layer, identified by staining sections with a monoclonal antibody against muscle-specific desmin (Dako, Ca). Staining was performed by immunoperoxidase technique using an Avidin/Biotin Blocking Kit, a rat-absorbed biotinylated anti-mouse IgG, and a

30 peroxidase-conjugate (all Vector Laboratories Burlingame, CA). Capillary density was determined at 2 weeks post infarction from sections labeled with anti-CD31 mAb, and confirmed with anti-factor VIII mAb, and compared to the capillary density of the unimpaired myocardium. Values are

expressed as the number of CD31-positive capillaries per HPF (400x).

Quantitation of cardiomyocyte proliferation: Cardiomyocyte
5   DNA synthesis and cell cycling was determined by dual staining of rat myocardial tissue sections obtained from LAD-ligated rats at two weeks after injection of either saline or CD34+ human cells, and from healthy rats as negative controls, for cardiomyocyte-specific troponin I and
10 human- or rat-specific Ki-67.   Briefly, paraffin embedded sections were microwaved in a 0.1M EDTA buffer, and stained with either a primary monoclonal antibody against rat Ki-67 at 1:3000 dilution (gift of Giorgio Catoretti, Columbia University) or human Ki-67 at 1:300 dilution (Dako, CA) and
15 incubated overnight at 4 degrees C.   Following washes, sections were incubated with a species-specific secondary antibody conjugated with alkaline phosphatase at 1:200 dilution (Vector Laboratories Burlingame, CA) for 30 minutes and positively-staining nuclei were visualized as blue with
20 a BCIP/NBT substrate kit (Dako, CA). Sections were then incubated overnight at 4 degrees C with a monoclonal antibody against cardiomyocyte-specific troponin I (Accurate Chemicals, CT) and positively-staining cells were visualized as brown through the Avidin/Biotin system described above.
25 Cardiomyocytes progressing through cell cycle in the infarct zone, peri-infarct region, and area distal to the infarct were calculated as the proportion of troponin I-positive cells per high power field co-expressing Ki-67.

30 Measurement of myocyte apoptosis by DNA end-labeling of paraffin tissue sections: For in situ detection of apoptosis at the single cell level we used the TUNEL method of DNA end-labeling mediated by dexynucleotidyl transferase (TdT)

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(Boehringer Mannheim, Mannheim, Germany). Rat myocardial tissue sections were obtained from LAD-ligated rats at two weeks after injection of either saline or CD34+ human cells, and from healthy rats as negative controls. Briefly, tissues

5   were deparaffinized with xylene and rehydrated with graded dilutions of ethanol and two washes in phosphate-buffered saline (PBS). The tissue sections were then digested with Proteinase K (10μg/ml in Tris/HCL) for 30 minutes at 37°C. The slides were then washed 3 times in PBS and incubated

10   with 50μl of the TUNEL reaction mixture (TdT and fluorescein-labeled dUTP) and incubated in a humid atmosphere for 60 minutes at 37°C. For negative controls TdT was eliminated from the reaction mixture. Following 3 washes in PBS, the sections were then incubated for 30

15   minutes with an antibody specific for fluorescein-conjugated alkaline phosphatase (AP) (Boehringer Mannheim, Mannheim, Germany). The TUNEL stain was visualized with a substrate system in which nuclei with DNA fragmentation stained blue, (BCIP/NBT substrate system, Dako, Carpinteria, CA). The

20   reaction was terminated following three minutes of exposure with PBS. To determine the proportion of blue-staining apoptotic nuclei within myocytes, tissue was counterstained with a monoclonal antibody specific for desmin. Endogenous peroxidase was blocked by using a 3% hydrogen perioxidase

25   solution in PBS for 15 minutes, followed by washing with 20% goat serum solution. An anti-troponin I antibody (Accurate Chemicals, CT) was incubated overnight (1:200) at 40 degrees C. Following 3 washes sections were then treated with an anti-rabbit IgG, followed by a biotin conjugated secondary

30   antibody for 30 minutes (Sigma, Saint Louis, Missouri). An avidin-biotin complex (Vector Laboratories, Burlingame, CA) was then added for an additional 30 minutes and the myocytes were visualized brown following 5 minutes exposure in DAB solution mixture (Sigma, Saint Louis, Missouri). Tissue

35   sections   were   examined   microscopically   at   200x

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magnification. Within each 200x field 4 regions were examined, containing at least 250 cells per region and cumulatively approximating 1 mm^2 of tissue, at both the peri-infarct site and distally to this site. Stained cells
5   at the edges of the tissue were not counted. Results were expressed as the mean number of apoptotic myocytes per mm^2 at each site examined.

Analysis of Myocardial Function: Echocardiographic studies
10   were performed using a high frequency liner array transducer (SONOS 5500, Hewlett Packard, Andover, MA). 2D images were obtained at mid-papillary and apical levels. End-diastolic (EDV) and end-systolic (ESV) left ventricular volumes were obtained by bi-plane area-length method, and % left
15   ventricular ejection fraction was calculated as [(EDV-ESV)/EDV] x 100.

CDNA Subtractive Hybridization: Briefly, messenger RNA was isolated from each heart, and 1 μg was used for first-strand
20   cDNA synthesis with random primers. The subtractive hybridization was performed with the PCR-select cDNA subtraction kit (CLONTECH), following the manufacturer's recommendations. After second-strand synthesis, the two cDNA libraries were digested with RsaI. Digestion products of the
25   "tester" library were ligated to a specific adapter (T7 promoter), then hybridized with a 30-fold excess of the "driver" library for subtraction. After hybridization, the remaining products were further amplified by PCR. In the forward subtraction, which determines the genes that are
30   overexpressed in the ischemic sample, the ischemic tissue is the "tester" and the normal tissue is the "driver." In the reverse subtraction, the "tester" and the "driver" are switched to determine the genes that are down-regulated in

the ischemic sample.

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Experimental Results II

<u>Intravenous Administration Of Human Bone Marrow-Derived
Endothelial Progenitor Cells Induces Neovascularization And</u>

5 <u>Prevents Cardiomyocyte Apoptosis Within Five Days
Post-Infarction</u>.

We sought to determine how soon after intravenous injection
of bone marrow-derived endothelial progenitor cells does

10 neovascularization develop in hearts of rats having
undergone permanent LAD ligation 48 hours earlier. When
animals were sacrificed at two days after intravenous
injection of DiI-labelled human CD34+ cells obtained by
G-CSF mobilization (>98% CD34 purity, containing 6-12%

15 CD117bright angioblasts), numerous DiI positive interstitial
cells were seen in the peri-infarct region, but no defined
vascular structures expressing DiI could be identified, data
not shown. In contrast, animals sacrificed at five days
post-infusion of human CD34+ cells demonstrated numerous

20 DiI-positive vascular structures at the peri-infarct region
and 3.5-fold higher numbers of capillaries in comparison to
rats receiving saline, figure 21(a) (p<0.01). The increase
in microvascularity at 5 days was accompanied by 3.3-fold
lower numbers of apoptotic cardiomyocytes at the

25 peri-infarct region, defined by dual positivity for troponin
I and TUNEL, in comparison to controls receiving saline,
figure 21(b) (p<0.01). Together, these data indicate that
the vasculogenic process of differentiation and organization
of bone marrow-derived angioblasts to a mature, functional

30 network of capillaries in the ischemic myocardium takes from
two to five days.

<u>Intravenous Administration Of Human Bone Marrow-Derived
Endothelial Progenitor Cells Induces Cell Cycling Of</u>

Cardiomyocyte Progenitors And Cardiomyocyte Differentiation.

We examined cardiac tissue from experimental and control
5   animals sacrificed at five days by immunohistochemistry and
confocal microscopy for evidence of cycling cardiomyocytes,
as has been suggested to occur rarely in the adult heart
after acute ischemia.   While no mature troponin-positive
cycling cardiomyocytes were detected in any control or
10   experimental animals at day 5 post-infarction, animals
receiving human bone marrow-derived CD34+ cells demonstrated
numerous   clusters   of   small,   cycling   cells   at   the
peri-infarct region that were of rat origin, as defined by
a monoclonal antibody specific for rat Ki67, figure 22(a).
15   These   cells   were   negative   for   cardiomyocyte-specific
troponin I, a marker of cardiomyocyte differentiation, but
stained positively for alpha-sarcomeric actin, indicating
they were immature cells of cardiomyocyte lineage.   Similar
clusters of cycling cardiomyocyte progenitors were not seen
20   in control animals injected with saline.

Tissues obtained from animals sacrificed at two weeks after
human CD34+ administration no longer demonstrated clusters
of small, cycling cardiomyocyte progenitors, but instead a
25   high frequency of large, mature rat cardiomyocytes at the
peri-infarct   region   with   detectable   DNA   activity,   as
determined by dual staining with mAbs reactive against
cardiomyocyte-specific troponin I and rat Ki67, figures 22
(b) and (c).   The number of cycling, mature cardiomyocytes
30   at the peri-infarct region was 4-fold higher in animals
receiving   human   endothelial   progenitor   cells   than   in
LAD-ligated controls receiving saline (p<0.01) in whom there
was a high frequency of cells with fibroblast morphology and

reactivity with rat Ki67, but not troponin I. We speculate that the cycling, mature cardiomyocytes seen at day 14 post-infarction in animals who received human endothelial progenitor cells are the differentiated progeny of the small, cycling immature cardiomyocyte progenitors seen in the same anatomical location at day 5 and accompanying the onset of neovascularization. Whether the cycling cardiomyocyte progenitors represent in situ cardiac stem cells normally residing in the heart in a quiescent state, or whether these cells have migrated to the heart from elsewhere in the body, such as the bone marrow, remains to be determined.

Myocardial Expression Of HBP23, A Rat Peroxiredoxin Protecting Cells Against Damage By Oxygen Radicals, Is Decreased By Ischemia And Increased By Neovascularization. We next sought to identify a molecular mechanism to explain the relationship between neovascularization at the peri-infarct region and proliferation/regeneration of adjacent cardiomyocyte progenitors. We first performed cDNA subtractive hybridization in order to identify patterns of changes in gene expression between normal rat hearts and rat hearts 48 hours after LAD ligation. Since deficits in antioxidants and increased oxidative stress accompanying myocardial infarction have been directly implicated in the pathogenesis of post-infarct heart failure (13-15), we chose to examine changes in expression of particular antioxidant genes. A family of antioxidant enzymes known as peroxiredoxins, which demonstrate peroxidase activity (16) and are induced by oxygen (17), play a critical role in regulating cell survival during periods of oxidative stress, such as ischemia. They serve to maintain the thiol-disulfide status of the cytosol by undergoing reversible oxidation-reduction reactions involving electron

transfer via disulfide bridges formed with thioredoxin (TRX), which constitutes one of the principal cellular reducing systems in mammals (18). In addition, peroxiredoxins can inhibit c-Abl tyrosine kinase activity
5   induced by oxidative stress (19,20) and can rescue cells from both a pro-apoptotic state and cell cycle arrest induced by the activated c-Abl gene product (21). By cDNA subtractive hybridization, we found that expression of the rat peroxiredoxin HBP23 mRNA was reduced in rat hearts 48
10   hours after LAD ligation compared with normal rat hearts. By RT-PCR, HBP23 mRNA levels in rat hearts decreased at two weeks post-LAD ligation by a mean of 34% compared with normal rat hearts, figure 23 (p<0.01). In contrast, HBP23 mRNA levels in LAD-ligated rat hearts two weeks after
15   receiving human angioblasts returned to levels only 14% lower than in non-ischemic controls. Since mRNA expression of peroxiredoxins is induced by oxygen, these data suggested that HBP23 mRNA expression was inhibited by the acute ischemic event and induced following angioblast-dependent
20   neovascularization.

Generation Of A DNA Enzyme To Cleave HBP23 mRNA. To investigate whether induced expression of HBP23 is involved in the mechanism by which neovascularization affects
25   myocardial cellular apoptosis, regeneration/proliferation, and function, we generated a catalytic DNA enzyme targeting specific sequences within the rat HBP23 gene (22,23). We chose to specifically target pyrimidine-purine junctions at or near the translational start site AUG of messenger RNA
30   for rat HBP23, a region that is conserved between species and has low relative free energy (24). In this region, the rat HBP23 sequence differs by only one base from the human homologue, proliferation-associated gene (PAG). To produce the control DNA enzyme, the nucleotide sequence in the two

flanking arms of the HBP23 DNA enzyme was scrambled without altering the catalytic domain. The 3' terminus of each molecule was capped with an inverted 3'-3'-linked thymidine for resistance to 3'-to-5' exonuclease digestion.

5

The DNA enzyme against HBP23 cleaved the 23-base oligonucleotide synthesized from the sequence of rat HBP23 mRNA, in a dose- and time-dependent manner, figure 24 (a). In contrast, the DNA enzyme did not cleave a 23-base

10   oligonucleotide form the human homologue PAG which differs from the rat HBP23 oligonucleotide by only one base, demonstrating its exquisite target specificity. A DNA enzyme with specificity for the same translational start site in the human PAG gene efficiently cleaved the PAG

15   oligonucleotide, but not the one derived from HBP23 (data not shown). The scrambled control DNA enzyme did not cleave either oligonucleotide. To determine the effect of the DNA enzyme on endogenous HBP23 production, cardiomyocyte monolayers obtained from fetal rat hearts were grown to

20   confluence and transfected with species-specific DNA enzymes or scrambled control. Densitometric analysis of RT-PCR products following reverse transcription of cellular mRNA showed that the HBP23 DNA enzyme inhibited steady-state mRNA levels in cultured rat cells by over 80%, figure 24 (b),

25   relative to the scrambled DNA.

<u>In Vivo Administration Of A DNA Enzyme To Prevent Induction</u>
<u>Of HBP23 mRNA In Rat Myocardium: Neovascularization Is Not</u>
<u>Affected, But Its Effects On Cardiomyocyte Apoptosis,</u>
30   <u>Regeneration, And Function Are Abrogated.</u>

To investigate the in vivo relevance of induced expression of HBP23 in experimental myocardial infarction, 48 hours

after LAD ligation rats were injected with human bone marrow-derived endothelial progenitor cells intravenously together with intramyocardial injections of either HBP23 DNA enzyme or scrambled control. As shown in figure 25 (a),
5   HBP23 DNA enzyme had no effect on induction of neovascularization by human bone marrow angioblasts. When sacrificed at day 5 post-infusion, rats who received human endothelial progenitors, irrespective of whether HBP23 DNA enzyme or scrambled control was co-injected, demonstrated
10 increased myocardial capillary density in comparison to saline controls. In contrast, injection of the HBP23 DNA enzyme, but not the scrambled control, abrogated the anti-apoptotic effects of neovascularization, figure 25 (b), and the improvement in cardiac function, figure 25 (c).
15 Among animals receiving human endothelial progenitor cells and demonstrating myocardial neovascularization, treatment with HBP23 DNA enzyme resulted in 1.7-fold higher levels of cardiomyocyte apoptosis ($p < 0.01$) and 38% mean deterioration in cardiac function as assessed by echocardiography
20 ($p < 0.01$). In addition, the HBP23 DNA enzyme exerted striking effects on cardiomyocyte progenitor proliferation/regeneration. Whereas peri-infarct clusters of small rat cells expressing Ki67 and alpha-sarcomeric actin were easily detected in animals receiving angioblasts
25 together with the scrambled control DNA, none were identified in animals receiving the HBP23 DNA enzyme. These results clearly demonstrate that angioblast-dependent neovascularization protects cardiomyocytes against apoptosis and induces proliferation/regeneration of resident
30 cardiomyocyte lineage progenitors via pathways regulated by peroxiredoxin gene products.

DISCUSSION

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In this study we showed that neovascularization of ischemic myocardium by human bone marrow derived angioblasts results in both protection of mature cardiomyocytes at the peri-infarct region against apoptosis and stimulation of cardiomyocyte progenitors at the same site to enter cell cycle, proliferate and regenerate. Moreover, we showed that the mechanism by which angioblast-dependent neovascularization regulates cardiomyocyte survival and self-renewal appears to involve at least one family of genes involved in anti-apoptotic and pro-proliferative pathways accompanying oxidative stress, the peroxiredoxins. While oxygen tension positively regulates peroxiredoxin gene expression (17), whether this alone explains the positive effect of angioblast-mediated neovascularization on HBP mRNA expression is not clear. Since peroxiredoxin mRNA expression is induced by protein kinase C delta (25) it is possible that bone marrow angioblasts may be a source of extracellular signals regulating protein kinase C delta activation and consequently cell survival, such as FGF-1 (26).

Deficits in antioxidants and increased oxidative stress accompanying myocardial infarction have been directly implicated in the pathogenesis of post-infarct heart failure (13-15). Our study suggests that reduced levels of peroxiredoxins post-infarction may be of direct causality in the subsequent remodelling and failure of the left ventricle. The antioxidant effects of peroxiredoxins are coupled with the physiological electron donor activity of the TRX system (27-29). In addition to directly interacting with TRX, the peroxiredoxin gene products specifically bind the SH3 domain of c-Abl, a non-receptor tyrosine kinase, inhibiting its activation (21). Activation of c-Abl through the SH3 domain by stimuli such as agents that damage DNA

induces either arrest of the cell cycle in phase G1 or cellular apoptosis (30). Cell cycle arrest is dependent on the kinase activity of c-Abl which downregulates the activity of the cyclin-dependent kinase Cdk2 and induces the

5   expression of p21 (31). By associating with c-Abl in vivo, peroxiredoxins can inhibit tyrosine phosphorylation induced by c-Abl overexpression and rescue cells from both the cytostatic and pro-apoptotic effects of the activated c-Abl gene product (32). Our in vivo demonstration that

10   co-administration of a DNA enzyme directed against the rat peroxiredoxin HBP23 abrogated the anti-apoptotic and pro-proliferative effects of human angioblast-dependent neovascularization in the infarcted rat heart argues strongly that this family of genes is directly implicated

15   in the mechanism of action by which neovascularization results in improvement in cardiac function and prevention of heart failure.

Throughout life, a mixture of young and old cells is present

20   in the normal myocardium. Although most myocytes seem to be terminally differentiated, there is a fraction of younger myocytes that retains the capacity to replicate (33). In the present study, human angioblast-dependent neovascularization resulted, within five days, in

25   proliferation of small, endogenous rat cardiomyocyte precursors at the peri-infarct region. The dividing myocyte precursors could be identified by immunohistochemical criteria on the basis of concomitant cell surface expression of alpha-sarcomeric actin, but not troponin I, and

30   proliferating nuclear structures, defined by an antibody specific for rat Ki67. Since this process was followed within fourteen days by increasing numbers in the same location of mature, cycling cardiomyocytes, defined on the basis of morphology, cell surface expression of troponin I,

and nuclear expression of Ki67, we conclude that cycling cardiomyocyte precursors differentiated in situ to become new, mature, functional cardiomyocytes. Whether these . precursors are derived from a resident pool of cardiomyocyte

5   stem cells or from a renewable source of circulating bone marrow-derived stem cells that home to the damaged myocardium remains to be determined. Moreover, while the signals required for in situ expansion of cardiomyocyte precursors appear to involve, at least in part, pathways

10   regulated by members of the peroxiredoxin gene family, the signals required for cardiomyocyte differentiation are, at present, unknown. Gaining an understanding of these issues may open the possibility of manipulating the biology of endogenous cardiomyocytes in order to augment the healing

15   process after myocardial ischemia.

METHODS AND MATERIALS

Purification and characterization of cytokine-mobilized

20   *human CD34+ cells.* Single-donor leukopheresis products were obtained from humans treated with recombinant G-CSF 10 mg/kg (Amgen, CA) sc daily for four days. Donors were healthy individuals undergoing standard institutional procedures of bone marrow mobilization, harvesting and isolation for

25   allogeneic stem cell transplants. Mononuclear cells were separated by Ficoll-Hypaque, and highly-purified CD34+ cells (>98% positive) were obtained using magnetic beads coated with anti-CD34 monoclonal antibody (mAb) (Miltenyi Biotech, CA). Purified CD34 cells were stained with

30   fluorescein-conjugated mAbs against CD34 and CD117 (Becton Dickinson, CA), AC133 (Miltenyi Biotech, CA), CD54 (Immunotech, CA), CD62E (BioSource, MA), VEGFR-2, Tie-2, vWF, eNOS, CXCR1, CXCR2, and CXCR4 (all Santa Cruz Biotech,

CA), and analysed by four-parameter fluorescence using
FACScan (Becton Dickinson, CA). Cells positively selected
for CD34 expression were also stained with phycoerythrin
(PE)-conjugated anti-CD117 mAb (Becton Dickinson, CA), and
5   sorted for bright and dim fluorescence using a Facstar Plus
(Becton Dickinson) and a PE filter. Intracellular staining
for GATA-2 was performed by permeabilizing one million cells
from each of the brightly and dimly fluorescing cell
populations using a Pharmingen Cytofix/Cytoperm kit,
10   incubating for 30 minutes on ice with 10ul of
fluorochrome-conjugated mAbs against both CD117 and CD34
surface antigens (Becton Dickinson, CA). After resuspension
in 250ul of Cytofix/Cytoperm solution for 20 minutes at 4
degrees C, cells were incubated with a fluorochrome-labeled
15   mAb against GATA-2 (Santa Cruz Biotech, CA) or IgG control
for 30 minutes at 4 degrees C, and analyzed by
three-parameter flow cytometry.

Animals, surgical procedures, and injection of human cells.
20   Rowett (rnu/rnu) athymic nude rats (Harlan Sprague Dawley,
Indianapolis, Indiana) were used in studies approved by the
"Columbia University Institute for Animal Care and Use
Committee". After anesthesia, a left thoracotomy was
performed, the pericardium was opened, and the left anterior
25   descending (LAD) coronary artery was ligated. Sham-operated
rats had a similar surgical procedure without having a
suture placed around the coronary artery.

Histology and measurement of infarct size. Following
30   excision at 2 and 15 weeks, left ventricles from each
experimental animal were sliced at 10-15 transverse sections
from apex to base. Representative sections were fixed in
formalin and stained for routine histology (H&E) to

determine cellularity of the myocardium, expressed as cell number per high power field (HPF) (600x). A Masson trichrome stain was performed, which labels collagen blue and myocardium red, to evaluate collagen content on a semiquantitative scale (0-3+), with 1+ light blue, 2+ light blue and patches of dark blue, and 3+ dark blue staining. This enabled measurement of the size of the myocardial scar using a digital image analyzer. The lengths of the infarcted surfaces, involving both epicardial and endocardial regions, were measured with a planimeter digital image analyzer and expressed as a percentage of the total ventricular circumference. Final infarct size was calculated as the average of all slices from each heart. All studies were performed by a blinded pathologist (MJS). Infarct size was expressed as percent of total left ventricular area. Final infarct size was calculated as the average of all slices from each heart.

Quantitation of capillary density. In order to quantitate capillary density and species origin of the capillaries, additional sections were stained freshly with mAbs directed against rat or human CD31 (Serotec, UK, and Research Diagnostics, NJ, respectively), factor VIII (Dako, CA), and rat or human MHC class I (Accurate Chemicals, CT). Arterioles were differentiated from large capillaries by the presence of a smooth muscle layer, identified by staining sections with a monoclonal antibody against muscle-specific desmin (Dako, Ca). Staining was performed by immunoperoxidase technique using an Avidin/Biotin Blocking Kit, a rat-absorbed biotinylated anti-mouse IgG, and a peroxidase-conjugate (all Vector Laboratories Burlingame, CA). Capillary density was determined at 5 days and 2 weeks post infarction from sections labeled with anti-CD31 mAb, and confirmed with anti-factor VIII mAb, and compared to the

capillary density of the unimpaired myocardium. Values are expressed as the number of CD31-positive capillaries per HPF (400x).

5   *Quantitation of cardiomyocyte proliferation.* Cardiomyocyte DNA synthesis and cell cycling was determined by dual staining of rat myocardial tissue sections obtained from LAD-ligated rats at two weeks after injection of either saline or CD34+ human cells, and from healthy rats as
10   negative controls, for cardiomyocyte-specific troponin I and human- or rat-specific Ki67. Briefly, paraffin embedded sections were microwaved in a 0.1M EDTA buffer, and stained with either a primary monoclonal antibody against rat Ki67 at 1:3000 dilution (gift of Giorgio Catoretti, Columbia
15   University) or human Ki67 at 1:300 dilution (Dako, CA) and incubated overnight at 4 degrees C. Following washes, sections were incubated with a species-specific secondary antibody conjugated with alkaline phosphatase at 1:200 dilution (Vector Laboratories Burlingame, CA) for 30 minutes
20   and positively-staining nuclei were visualized as blue with a BCIP/NBT substrate kit (Dako, CA). Sections were then incubated overnight at 4 degrees C with a monoclonal antibody against cardiomyocyte-specific troponin I (Accurate Chemicals, CT) and positively-staining cells were visualized
25   as brown through the Avidin/Biotin system described above. Cardiomyocytes progressing through cell cycle in the infarct zone, peri-infarct region, and area distal to the infarct were calculated as the proportion of troponin I-positive cells per high power field co-expressing Ki67. For confocal
30   microscopy, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG was used as secondary antibody to detect Ki67 in nuclei. A Cy5-conjugated mouse mAb against alpha-sarcomeric actin (clone 5C5; Sigma) was used to detect cardiomyocytes, and propidium iodide was used to identify

all nuclei.

*Measurement of myocyte apoptosis by DNA end-labeling of
paraffin tissue sections.*   For in situ detection of
apoptosis at the single cell level we used the TUNEL method
of DNA end-labeling mediated by dexynucleotidyl transferase
(TdT) (Boehringer Mannheim, Mannheim, Germany).   Rat
myocardial tissue sections were obtained from LAD-ligated
rats at two weeks after injection of either saline or CD34+
human cells, and from healthy rats as negative controls.
Briefly, tissues were deparaffinized with xylene and
rehydrated with graded dilutions of ethanol and two washes
in phosphate-buffered saline (PBS).   The tissue sections
were then digested with Proteinase K (10ug/ml in Tris/HCL)
for 30 minutes at 370 C.   The slides were then washed 3
times in PBS and incubated with 50ul of the TUNEL reaction
mixture (TdT and fluorescein-labeled dUTP) and incubated in
a humid atmosphere for 60 minutes at 370 C.   For negative
controls TdT was eliminated from the reaction mixture.
Following 3 washes in PBS, the sections were then incubated
for   30   minutes   with   an   antibody   specific   for
fluorescein-conjugated alkaline phosphatase (AP) (Boehringer
Mannheim,   Mannheim,   Germany).   The TUNEL stain was
visualized with a substrate system in which nuclei with DNA
fragmentation stained blue, (BCIP/NBT substrate system,
DAKO, Carpinteria, CA).   The reaction was terminated
following three minutes of exposure with PBS. To determine
the proportion of blue-staining apoptotic nuclei within
myocytes, tissue was counterstained with a monoclonal
antibody specific for desmin.   Endogenous peroxidase was
blocked by using a 3% hydrogen perioxidase solution in PBS
for 15 minutes, followed by washing with 20% goat serum
solution. An anti-troponin I antibody (Accurate Chemicals,
CT) was incubated overnight (1:200) at 40 degrees C.

Following 3 washes sections were then treated with an anti-rabbit IgG, followed by a biotin conjugated secondary antibody for 30 minutes (Sigma, Saint Louis, Missouri). An avidin-biotin complex (Vector Laboratories, Burlingame, CA)

5   was then added for an additional 30 minutes and the myocytes were visualized brown following 5 minutes exposure in DAB solution mixture (Sigma, Saint Louis, Missouri). Tissue sections were examined microscopically at 20X magnification. Within each 20X field 4 regions were examined, containing

10   at least 250 cells per region and cumulatively approximating 1 mm2 of tissue, at both the peri-infarct site and distally to this site. Stained cells at the edges of the tissue were not counted. Results were expressed as the mean number of apoptotic myocytes per mm2 at each site examined.

15

Analyses of myocardial function: Echocardiographic studies were performed using a high frequency liner array transducer (SONOS 5500, Hewlett Packard, Andover, MA). 2D images were obtained at mid-papillary and apical levels. End-diastolic

20   (EDV) and end-systolic (ESV) left ventricular volumes were obtained by bi-plane area-length method, and % left ventricular ejection fraction was calculated as [(EDV-ESV)/EDV] x 100.

25   *cDNA Subtractive Hybridization:* This technique enabled comparison of the pattern of gene expression between hearts from normal rats and rats who underwent left anterior descending (LAD) coronary artery ligation 48 hours earlier. Briefly, messenger RNA was isolated from each heart, and 1

30   µg was used for first-strand cDNA synthesis with random primers. The subtractive hybridization was performed with the PCR-select cDNA subtraction kit (CLONTECH), following the manufacturer's recommendations. After second-strand

synthesis, the two cDNA libraries were digested with RsaI.
Digestion products of the "tester" library were ligated to
a specific adapter (T7 promoter), then hybridized with a
30-fold excess of the "driver" library for subtraction.

5   After hybridization, the remaining products were further
amplified by PCR.   In the forward subtraction, which
determines the genes that are overexpressed in the ischemic
sample, the ischemic tissue is the "tester" and the normal
tissue is the "driver."   In the reverse subtraction, the

10   "tester" and the "driver" are switched to determine the
genes that are down-regulated in the ischemic sample.

*Reverse transcription-polymerase chain reaction (RT-PCR)
analysis of HBP23 mRNA expression:* Total RNA was extracted

15   using RNeasy Kits from Qiagen (Valencia, CA) from normal rat
hearts or from hearts of rats who underwent LAD ligation two
weeks earlier and received either saline or human
angioblasts. RNA was reverse transcribed with SMART cDNA
Synthesis Kit (Clontech, Palo Alto, CA). Amplification

20   reactions were conducted in a 25ul volume with an initial
step of 94C for 5 min, followed by 26-32 cycles of 94C for
30 sec and 68C for 1 min, using TITANIUM Taq PCR Kits
(Clontech, Palo Alto, CA). Primers for HBP23 were
5'-GCTGATGAAGGTATCTCTTTCAGGGGCCTC (SEQ ID NO:10) and

25   5'-GATGGTCTGCCCCTTACCAATAGTGGAAG (SEQ ID NO:11). Rat GAPDH
was used as internal control (forward primer
5'TGAAGGTCGGAGTCAACGGATTTG3' (SEQ ID NO:12), reverse primer
5'CATGTGGGCCA TGAGG TCCA CCAC3' (SEQ ID NO:13)). Ethidium
bromide stained bands of amplified fragments were quantified

30   by densitometric.

DNA enzymes and RNA substrates: DNA enzymes with 3'-3'
inverted thymidine were synthesized by Integrated DNA

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technologies (Coralville,IA)   and purified by RNase-free
IE-HPLC or RP-HPLC. The short RNA substrates corresponding
to target DNA enzyme sequences were chemically synthesized
followed by RNAse-free PAGE purification and also made by

5   in vitro transcription from a DNA template. Rat HBP23 cDNA
and human PAG cDNA were amplified by RT-PCR from total RNA
of   cultured   rat   fetal   cardiomyocytes   and   HUVEC,
respectively,   using   the   following   primer   pair:
5'TTTACCCTCTTGACTTTACTTTTGTGTGTCCCAC3' (forward primer) and

10   5'CCAGCTGGGCACACTTCACCATG3' (reverse primer). HBP23 and PAG
cDNA were cloned into pGEM-T vectors (Promega) to obtain
plasmid constructs pGEM-ratHBP23 and pGEM-humanPAG. cDNA
sequences   were   verified   using   an   automatic   sequencing
machine. 32P-labeled-nucleotide rat HBP23 and human PAG RNA

15   transcripts were prepared by in vitro transcription (SP6
polymerase, Promega) in a volume of 20ml for 1 hour at 32°C.
Unincorporated label and short nucleotides (<350base) were
separated from radiolabeled species by centrigugation on
Chromaspin-200 columns (Clontech, Palo Alto,CA). Synthetic

20   RNA   substrates   were   end-labeled   with   32P   using   T4
polynucletiode kinase and incubated with 0.05¾5uM HBP23 or
scrambled DNA enzyme. Reactions were allowed to proceed at
37°C and were "quenched " by transfer of aliquots to tubes
containing 90% formamide, 20mM EDTA and loading dye. Samples

25   were separated by electrophoresis on 15% TBE-urea denaturing
polyacrylamide gels and detected by autoradiography at
-80°C. Primary rat fetal cardiomyocytes were obtained from
Clonetic (USA) and grown in medium containing 2% FCS,
100ug/ml stretomycin and 100 IU/ml penicillin at 37°C in a

30   humidified atmosphere of 5% CO_2. Cells were used in
experiments between passage 6 and 8. Subconfluent (70~80%)
rat fetal cardiomyocytes were transfected using 0.5ml of
serum-free medium containing 0.05¾5uM HBP23 or scrambled DNA
enzyme and 20 ug/ml cationic lipids (DOTAP).   After

35   incubation for eight hours cells were lysed using Trizol

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reagent (LifeSciences, CA) to isolate RNA for RT-PCR of HBP23 expression, as above.

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What is claimed is:

1.   A method of treating a disorder of a subject's heart
      involving loss of cardiomyocytes which comprises
5   administering to the subject an amount of an agent
      effective to cause cardiomyocyte proliferation within
      the subject's heart so as to thereby treat the
      disorder.

10   2.   The method of claim 1, wherein the agent is human
      endothelial progenitor cells.

3.   The method of claim 2 which further comprises
      administering an effective amount of a second agent
15   that increases the cardiomyocyte proliferation caused
      by the human endothelial progenitor cells.

4.   The method of claim 3, wherein the agent is an
      antisense oligonucleotide which specifically inhibits
20   translation of Vitamin D3 Up-Regulated Protein-1
      (VDUP-1) mRNA.

5.   The method of claim 3, wherein the agent is a
      catalytic nucleic acid which specifically inhibits
25   translation of Vitamin D3 Up-Regulated Protein-1 mRNA.

6.   The method of claim 5, wherein the catalytic nucleic
      acid comprises deoxyribonucleotides.

30   7.   The method of claim 5, wherein the catalytic nucleic

acid comprises ribonucleotides.

8. The method of claim 3, wherein the second agent is a pro-angiogenic agent.

9. The method of claim 8, wherein the pro-angiogenic agent is vascular endothelial growth factor, fibroblast growth factor or angiopoietin.

10. The method of claim 3, wherein the second agent induces expression of a pro-angiogenic factor.

11. The method of claim 10, wherein the second agent is Hypoxia Inducible Factor-1.

12. The method of claim 3, wherein the second agent promotes trafficking of the endothelial progenitor cells to the subject's heart.

13. The method of claim 12, wherein the second agent that promotes trafficking is an antibody directed against an epitope of CXCR4.

14. The method of claim 12, wherein the second agent that promotes trafficking is a CC chemokine.

15. The method of claim 14, wherein the CC chemokine is RANTES, EOTAXIN, monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, or MCP.

16.   The method of claim 12, wherein the second agent is a
       CXC chemokine.

5   17.   The method of claim 16, wherein the CXC chemokine is
       Interleukin-8, Gro-Alpha, or Stromal-Derived Factor-1.

18.   The method of claim 17, wherein the Stromal-Derived
       Factor-1   is   Stromal-Derived   Factor-1   alpha   or
10   Stromal-Derived Factor-1 beta.

19.   The method of claim 3, wherein the second agent
       promotes mobilization of angioblasts into the
       bloodstream of the subject.

15

20.   The method of claim 19, wherein the agent is G-CSF,
       GM-CSF, SDF-1, or VEGF.

21.   The method of claim 3, wherein the second agent is
20   cardiomyocyte progenitor cells.

22.   The method of claim 3, wherein the second agent is
       skeletal muscle progenitor cells.

25   23.   The method of claim 2, wherein the effective amount of
       human endothelial progenitor cells is between 2.5×10^5
       and 7.5×10^5 endothelial progenitor cells per kg of the
       subject's body mass.

24.   The method of claim 23, wherein the effective amount is 5×10^5 endothelial progenitor cells per kg of the subject's body mass.

5   25.   The method of claim 2, wherein the endothelial progenitor cells are allogeneic with respect to the subject.

26.   The method of claim 25, wherein the subject is an
10   adult.

27.   The method of claim 25, wherein the subject is an embryo or a fetus.

15   28.   The method of claim 2, wherein the administering comprises injecting directly into the subject's peripheral circulation, heart muscle, left ventricle, right ventricle, coronary artery, cerebro-spinal fluid, neural tissue, ischemic tissue, or
20   post-ischemic tissue.

29.   The method of claim 2, wherein the human endothelial progenitor cells express CD117, CD34 or AC133.

25   30.   The method of claim 2, wherein endothelial progenitor cells express a high level of intracellular GATA-2 activity.

31.   The method of claim 1, wherein the agent induces
30   expression of a mRNA encoding a peroxiredoxin.

32.   The method of claim 31, wherein the agent is 2(3)-t-butyl-4-hydroxyanisole.

5   33.   The method of claim 1, wherein the agent induces expression of a mRNA encoding NF-E2-related factor 2 (Nrf2).

34.   The method of claim 1, wherein the agent induces
10   dissociation of a Nrf2 protein from a Keap-1.

35.   The method of claim 1, wherein the agent inhibits association of a Nrf2 protein with a Keap-1.

15   36.   The method of claim 1, wherein the agent inhibits association of a thiol reductase thioredoxin with a VDUP-1 protein.

37.   The method of claim 1, wherein the agent inhibits c-
20   Abl tyrosine kinase activation.

38.   The method of claim 37, wherein the agent is STI-571.

39.   The method of claim 1, wherein the agent is a CXC
25   chemokine.

40.   The method of claim 39, wherein the chemokine is Stromal-Derived Factor-1, Il-8 or Gro-Alpha.

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41. The method of claim 40, wherein the agent is
     Stromal-Derived Factor-1 and is administered
     intramyocardially.

5

42. The method of claim 40, wherein the agent is
     Stromal-Derived Factor-1 and is administered
     intracoronary.

10   43. The method of claim 40, wherein the agent is
          Stromal-Derived Factor-1 and is administered via a
          stent, a scaffold, or as a slow-release formulation.

44. The method of claim 39, further comprising
15   administering a second agent.

45. The method of claim 44, wherein the second agent is
     GM-CSF, G-CSF, IL-8 or a Gro family chemokine.

20   46. The method of claim 44, wherein the second agent is an
          inhibitor of CXCR4 or an inhibitor of SDF-1.

47. The method of claim 1, wherein the agent is an
     inhibitor of plasminogen activator inhibitor-1.

25

48. The method of claim 1, wherein the agent is an
     antibody directed against an epitope of CXCR4.

49. The method of claim 1, wherein the agent is G-CSF, GM-

CSF, an inhibitor of VDUP1 expression, or a Gro family chemokine.

50. The method of claim 1, wherein the subject has a cardiovascular disease.

51. The method of claim 50, wherein the subject has congestive heart failure.

52. The method of claim 50, wherein the subject has suffered a myocardial infarct.

53. The method of claim 50, wherein the subject has suffered myocardial ischemia.

54. The method of claim 50, wherein the subject has angina.

55. The method of claim 50, wherein the subject has a cardiomyopathy.

56. The method of claim 1, wherein the subject is a mammal.

57. The method of claim 56, wherein the mammal is a human being.

58. A method of determining the susceptibility of a

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cardiomyocyte in a subject to apoptosis comprising:

(a) quantitating the amount of mRNA encoding peroxiredoxin in the cardiomyocyte;

5

(b) quantitating the amount of mRNA encoding Vitamin D3 Up-Regulated Protein-1 in the cardiomyocyte; and

(c) determining the ratio of the amount of mRNA encoding peroxiredoxin: amount of mRNA encoding Vitamin D3 Up-Regulated Protein-1, wherein a low

10   ratio indicates a high susceptibility of the cardiomyocyte to apoptosis and a high ratio indicates a low susceptibility of the cardiomyocyte to apoptosis in the subject.

15   59. A method of determining the susceptibility of a cardiomyocyte in a subject to apoptosis comprising:

(a) quantitating the expression of a peroxiredoxin protein in the cardiomyocyte;

(b) quantitating the expression of Vitamin D3

20   Up-Regulated Protein-1 in the cardiomyocyte; and

(c) determining the ratio of the peroxiredoxin protein expression: Vitamin D3 Up-Regulated Protein-1 expression, wherein a low ratio indicates a high susceptibility of the

25   cardiomyocyte to apoptosis and a high ratio indicates a low susceptibility of the cardiomyocyte to apoptosis in the subject.

60. A cardiac progenitor cell.

30

61. A method of inducing cell cycling in a cardiac

progenitor cell in a heart tissue of a subject comprising administering to the subject an amount of an agent effective to induce neovascularization of the heart tissue in the subject so as to thereby induce cardiac cell cycling within the heart tissue of the subject.

62. A method of improving cardiac function in a subject comprising administering to the subject an amount of an agent effective to induce neovascularization of a heart tissue in the subject so as to thereby improve cardiac function in the subject.

63. A method of improving cardiac function in a subject comprising administering to the subject an amount of an agent effective to induce proliferation of cardiomyocytes in the heart tissue in the subject so as to thereby improve cardiac function in the subject.

64. The method of claim 62 or 63, wherein the subject has suffered myocardial ischemia or myocardial infarct.

65. The method of claim 62 or 63, wherein the agent is G-CSF, GM-CSF, IL-8, a Gro family chemokine, an inhibitor of CXCR4, or an inhibitor of SDF-1.

66. The method of claim 65, wherein the inhibitor of CXCR4 is a small molecule or a monoclonal antibody.

67. The method of claim 65, wherein the inhibitor of CXCR4 is a small molecule and is AMD 3100.

68.   The method of claim 65, wherein the inhibitor of CXCR4
        is AMD 070.

5   69.   The method of claim 65, wherein the inhibitor of CXCR4
        is a catalytic nucleic acid, an oligonucleotide, a
        monoclonal antibody, RNAi, or a small molecule.

70.   The method of claim 65, wherein the inhibitor of SDF-1
10   is a small molecule or a monoclonal antibody.

71.   The method of claim 65, wherein the Gro family
        chemokine is Gro alpha.

15   72.   A method of inducing apoptosis in a cell comprising
        inhibiting expression of a peroxiredoxin in the cell.

73.   The method of claim 72, wherein the cell expresses
        peroxiredoxin.

20

74.   The method of claim 73, wherein the cell is supplied
        by a vasculature, and the vasculature induces
        expression of the peroxiredoxin.

25   75.   The method of claim 72, wherein the peroxiredoxin is
        peroxiredoxin I, peroxiredoxin II, peroxiredoxin III,
        peroxiredoxin IV, or peroxiredoxin V.

76.   The method of claim 72, wherein the inhibition of

expression of peroxiredoxin is effected by contacting
the cell with a catalytic nucleic acid which binds
mRNA encoding peroxiredoxin, thereby inhibiting the
expression thereof.

5

77.   The method of claim 72, wherein the cell is a tumor
       cell.

78.   A method of treating a disorder of tissue cells of a
10   subject involving loss of tissue cells which comprises
       administering to the subject an amount of an agent
       effective to cause tissue cell proliferation within
       the tissue of the subject so as to thereby treat the
       disorder of tissue cells of a subject.

15

79.   The method of claim 78, wherein the agent is G-CSF,
       SDF-1, GM-CSF, IL-8, or VEGF.

80.   The method of claim 78, wherein the agent is an
20   inhibitor of CXCR4/SDF-1 interaction.

81.   The method of claim 80, wherein the inhibitor is a
       catalytic nucleic acid, a monoclonal antibody, a
       antisense oligonucleotide, a small molecule, or RNAi.

25

82.   The method of claim 78, wherein the agent is an
       inducer of peroxiredoxin expression.

83.   The method of claim 79 or 80, wherein the tissue is
30   cardiac tissue, brain tissue, peripheral vascular

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tissue, hepatic tissue, renal tissue, gastrointestinal tissue, lung tissue, smooth muscle tissue, or striated muscle tissue.

5   84.   The method of claim 20, 45, 65, or 79, wherein the agent is G-CSF, and the G-CSF is a covalent conjugate of is a covalent conjugate of recombinant methionyl human G-CSF and monomethoxypolyethylene glycol.

10   85.   A method of treating a disorder of a tissue of a subject involving apoptosis of cells in the tissue which comprises administering to the subject an amount of an agent effective to inhibit apoptosis of cells in the tissue within the subject so as to thereby treat

15   the disorder.

     86.   The method of claim 85, wherein the agent is an inhibitor of VDUP-1 expression.

20   87.   The method of claim 86, wherein the inhibitor of VDUP-1 expression is catalytic nucleic acid, a monoclonal antibody, an antisense oligonucleotide, a small molecule, or an RNAi.

25   88.   The method of claim 86, wherein the tissue is cardiac tissue, cerebrovascular tissue, or cerebral tissue.

     89.   A method of inhibiting proliferation of fibroblasts or inflammatory cells in a tissue and thereby

30   inhibiting collagen formation comprising contacting

the tissue with an amount of an inhibitor of VDUP-1 expression effective to inhibit proliferation of fibroblasts or inflammatory cells in the tissue.

5   90.   The method of claim 89, wherein the inhibitor of VDUP-1 expression is a catalytic nucleic acid, a monoclonal antibody, an antisense oligonucleotide, a small molecule, or an RNAi.

10

FIGURE 1A

FIGURE 1B

FIGURE 1C

saline   CD34   CD34 +
anti-CXCR1/2

trichrome

FIGURE 1D

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FIGURE 2A

Days post intravenous injection of CD34+ cells

FIGURE 2B

FIGURE 2C

FIGURE 3A

FIGURE 3B

FIGURE 3C

FIGURE 3D

FIGURE 3E

FIGURE 3F

FIGURE 4B

FIGURE 4A

FIGURE 4C

FIGURE 4D

FIGURE 4F

FIGURE 4E

FIGURE 4G

FIGURE 4H

left anterior wall

septum

10^3 angioblasts

left anterior wall

septum

$2x10^5$ angioblasts
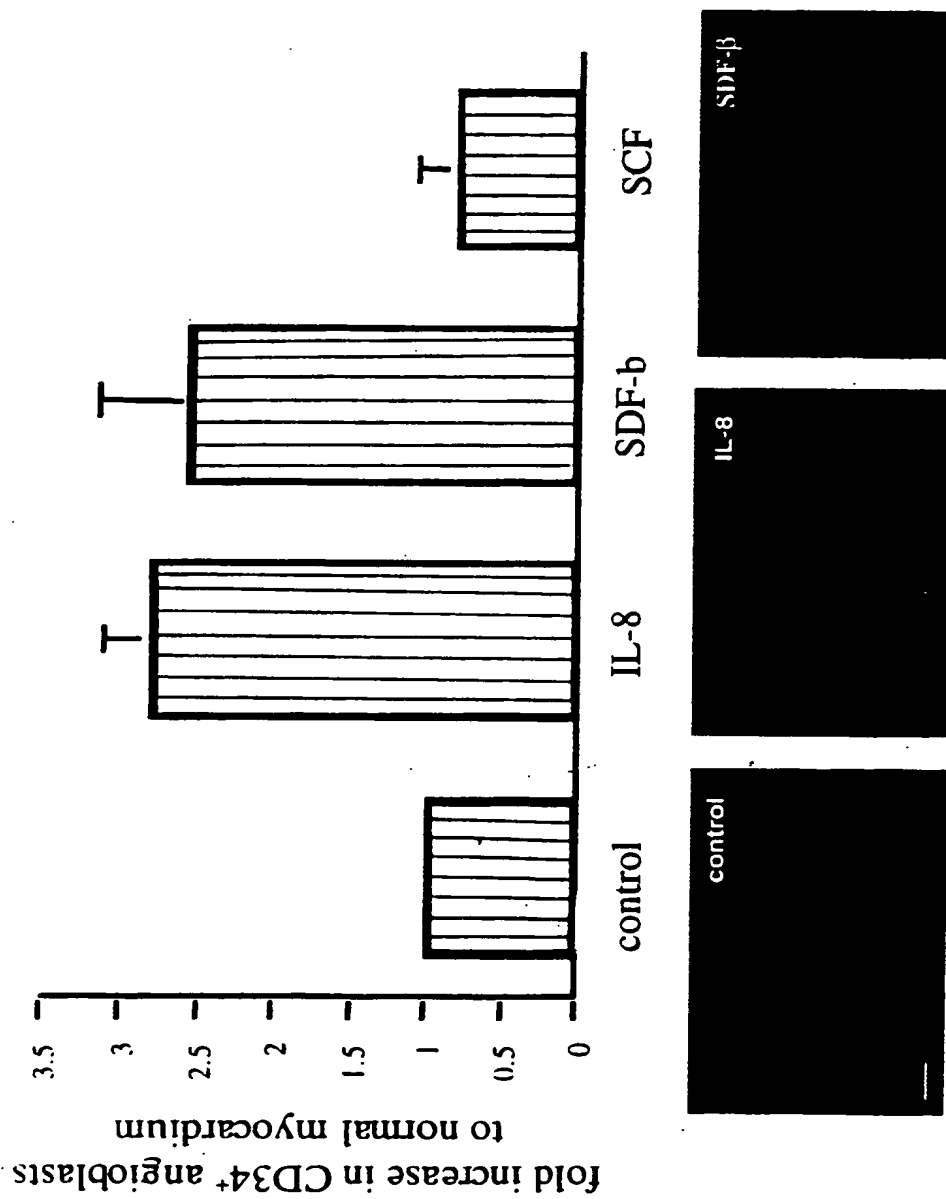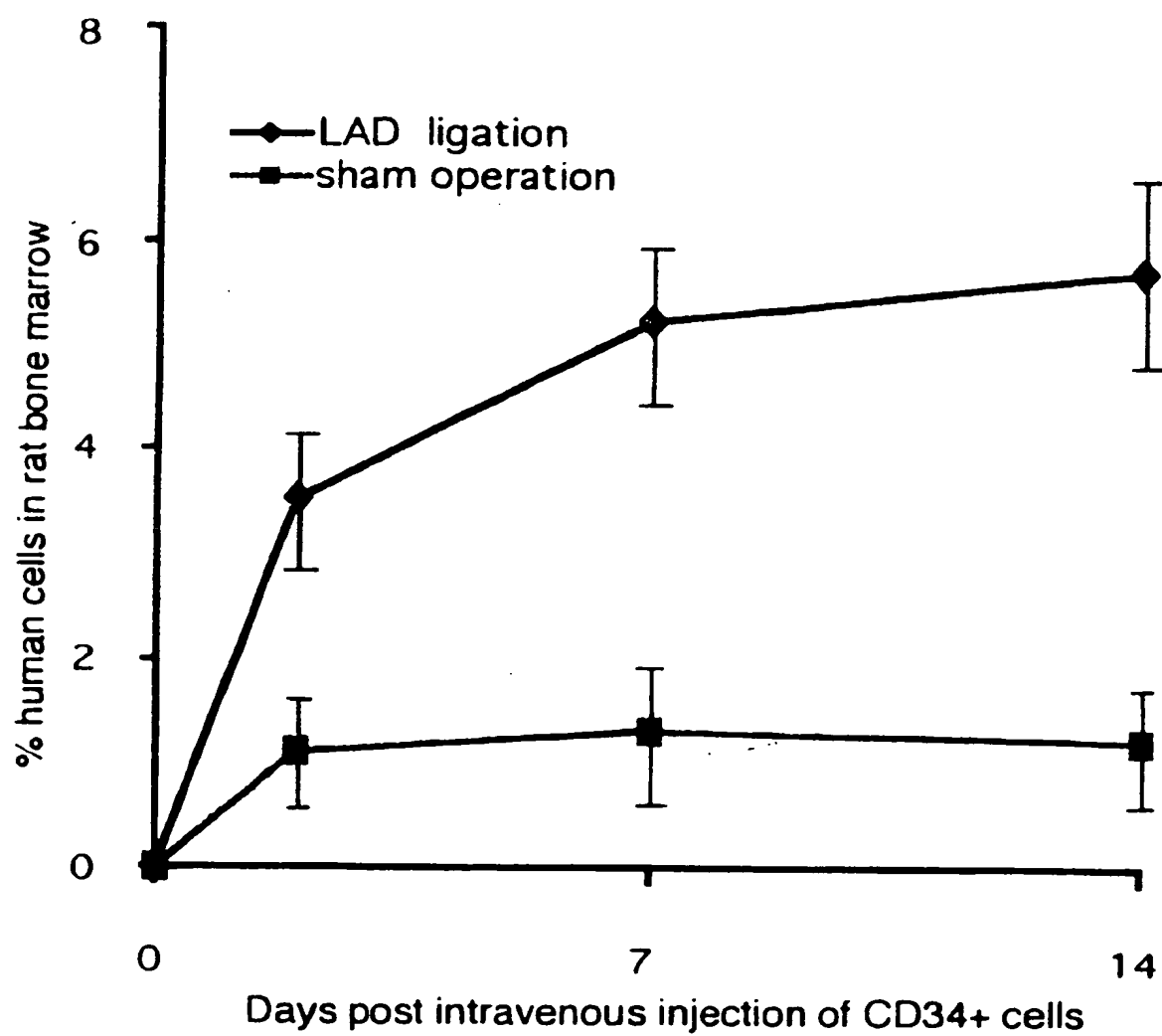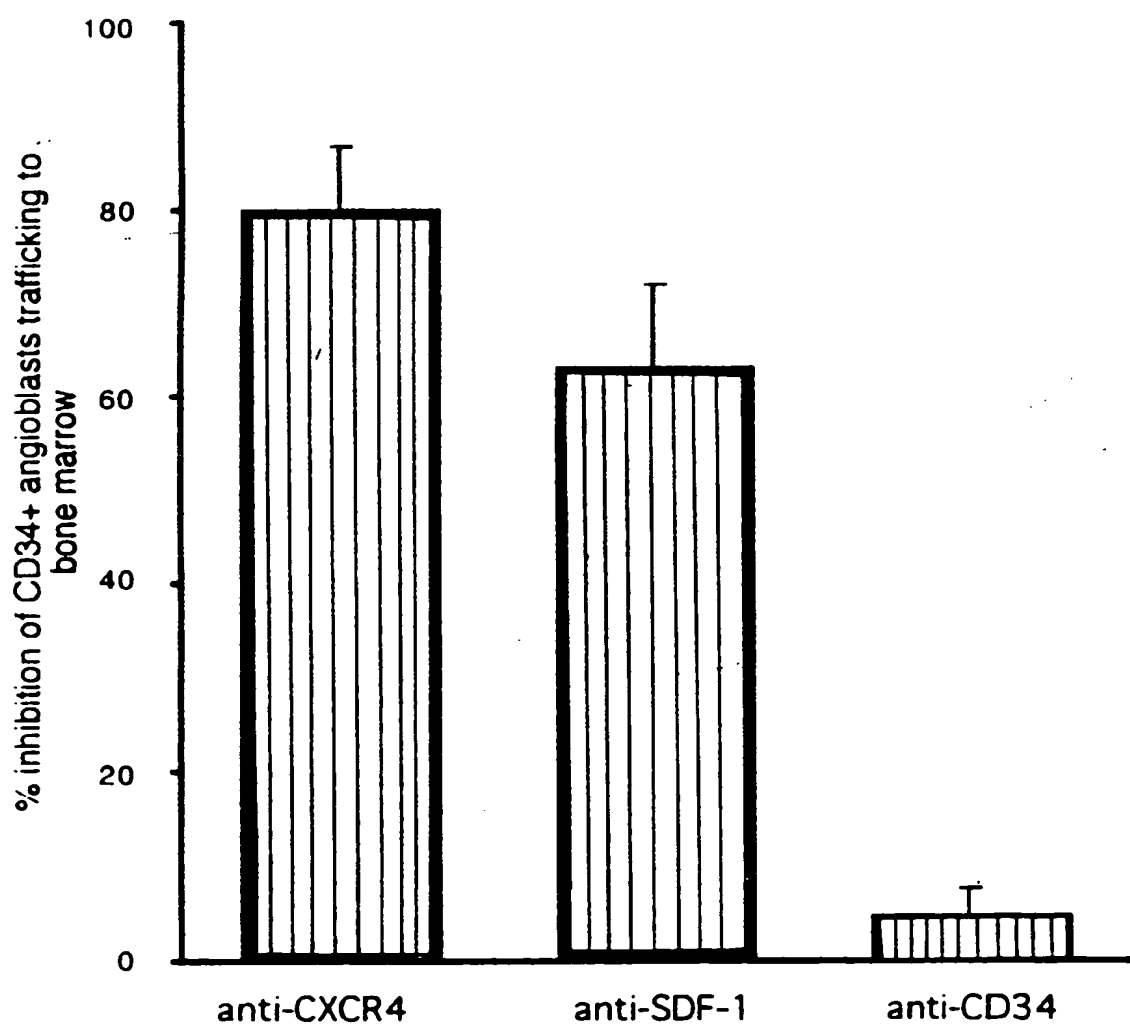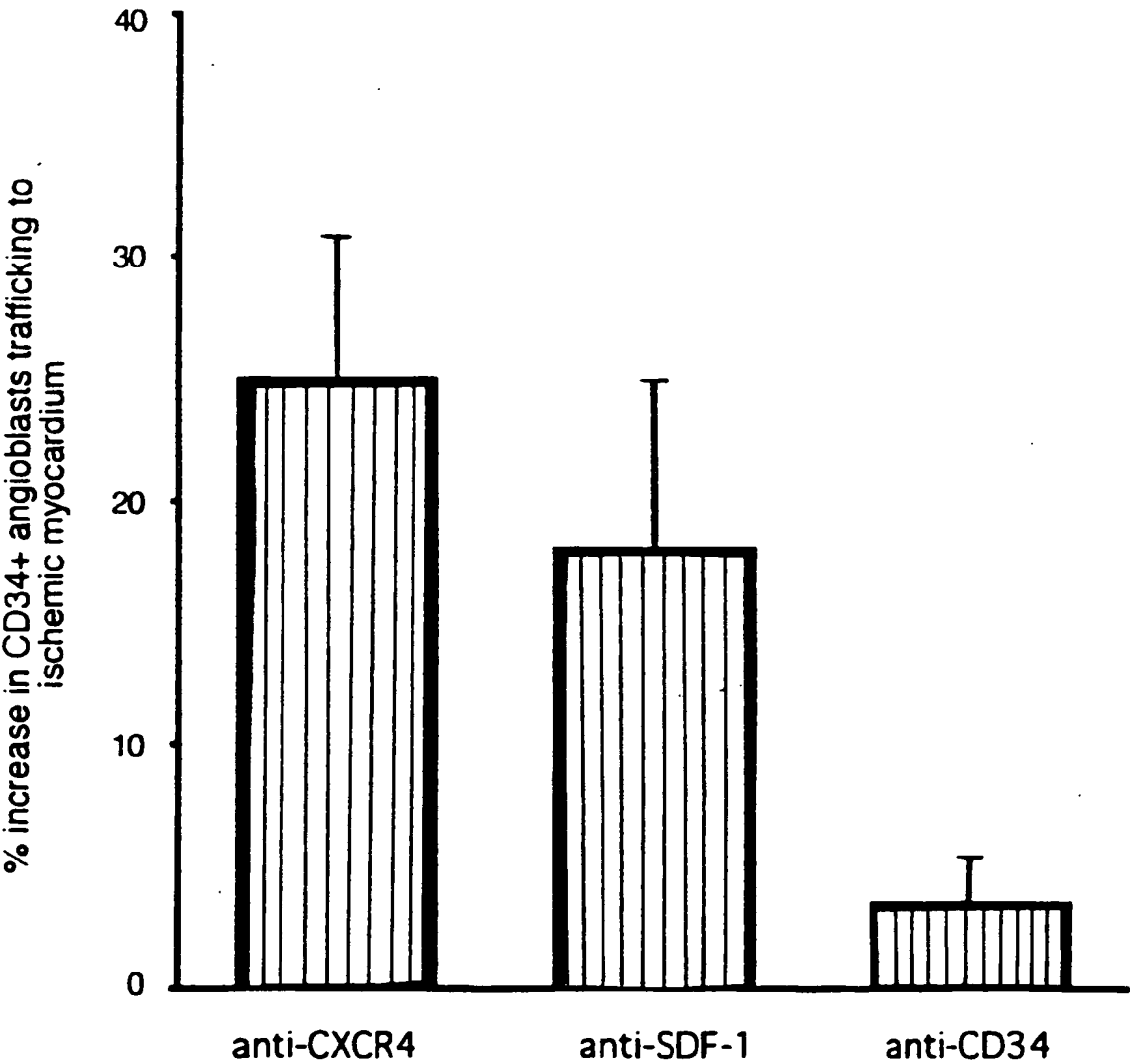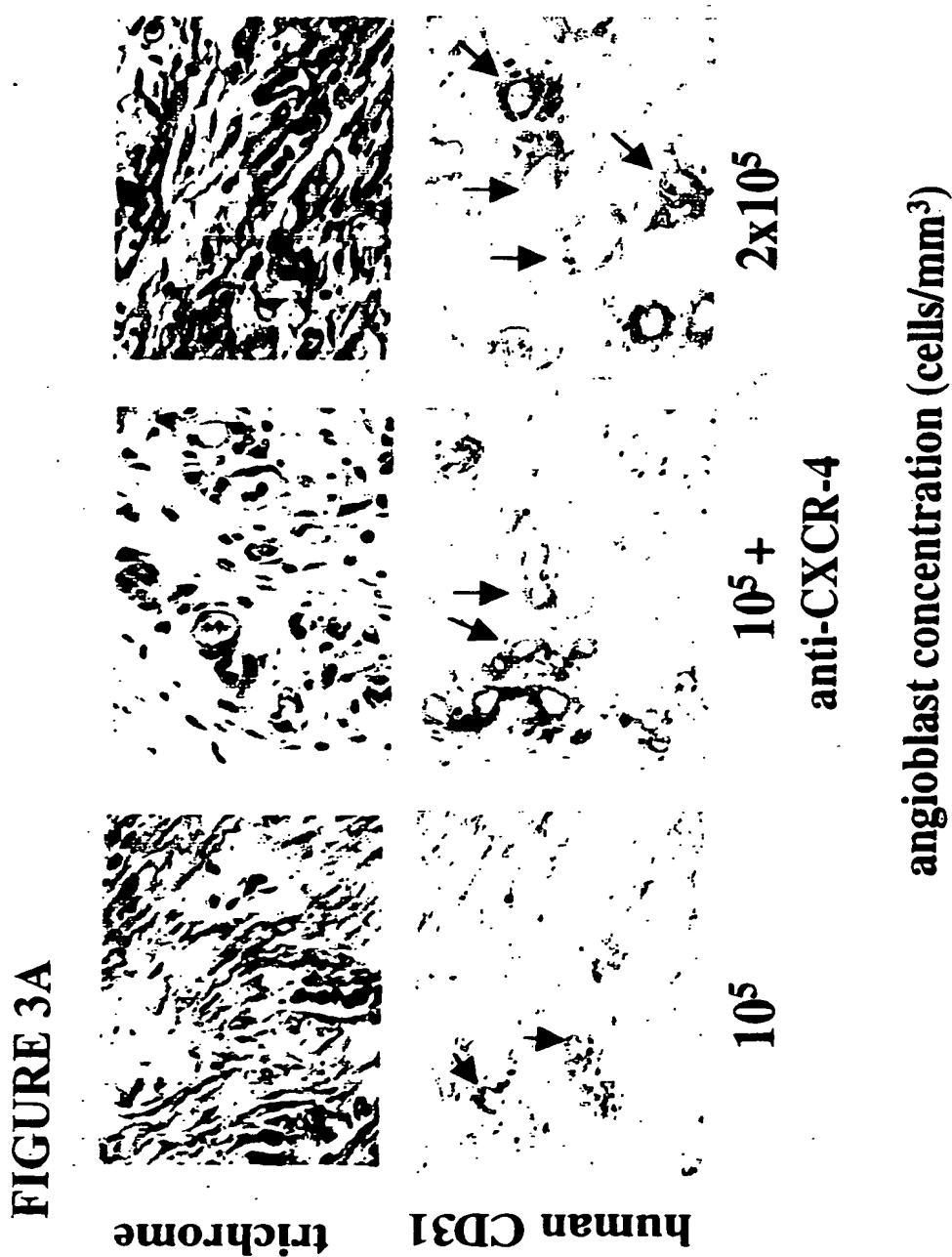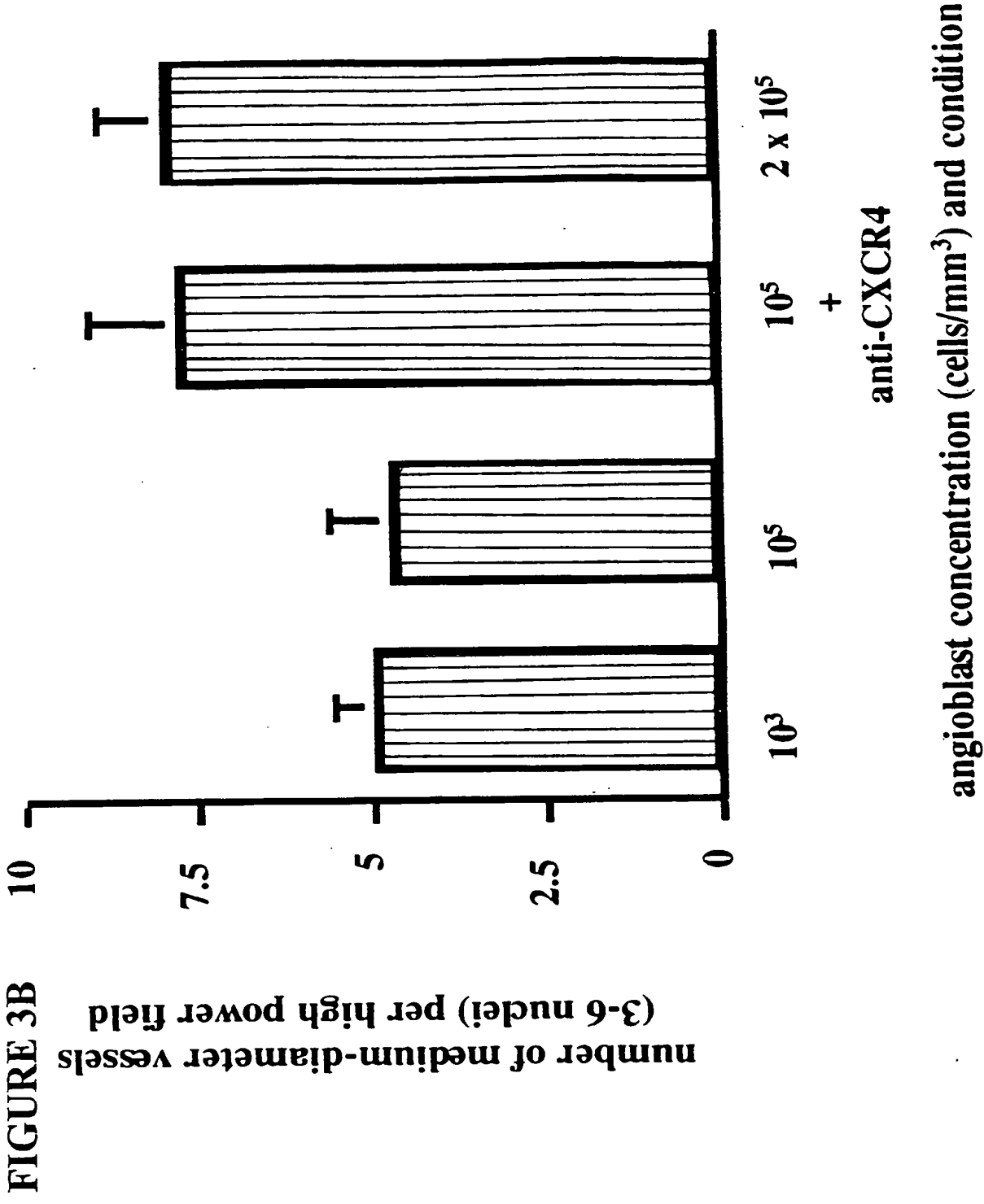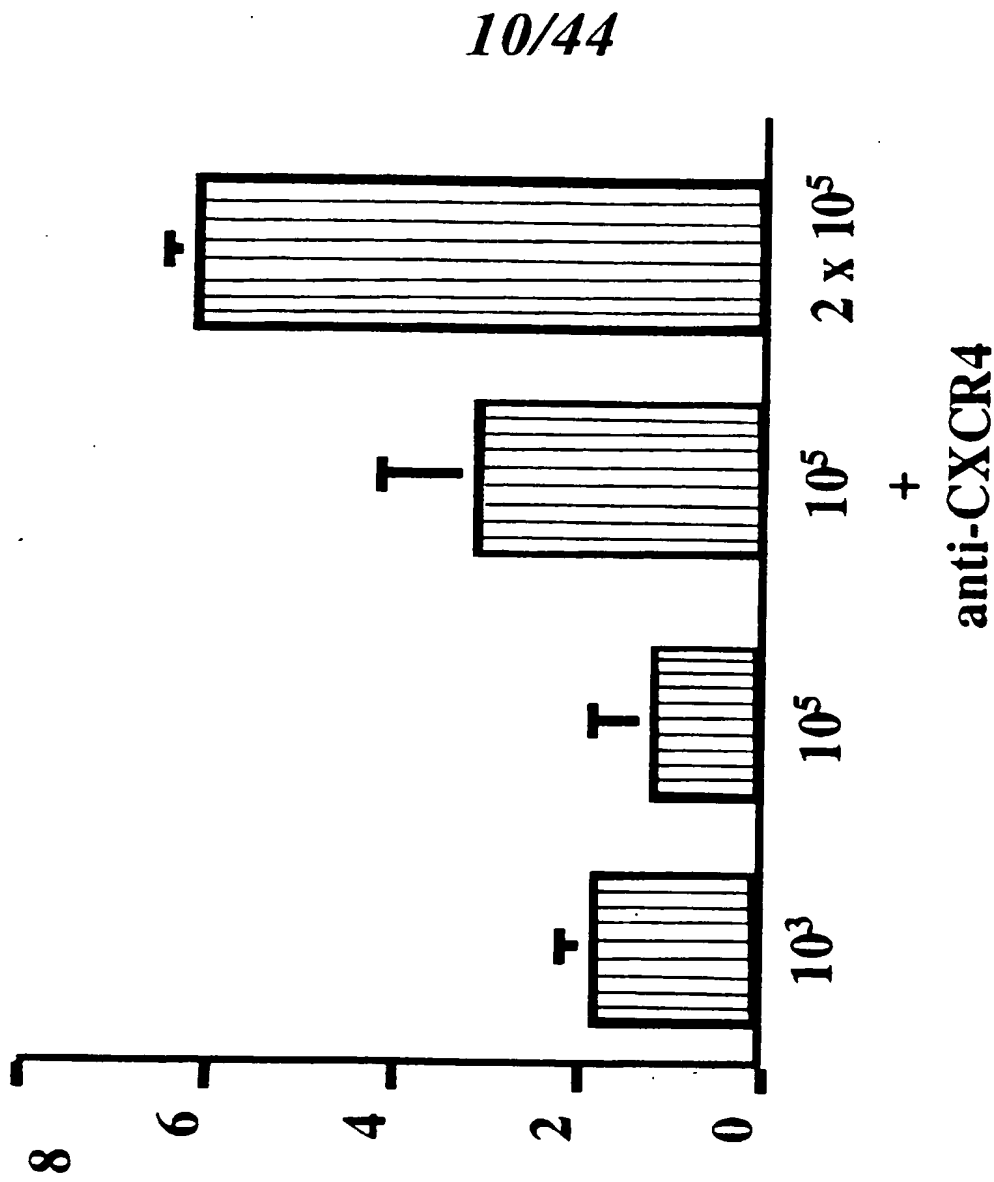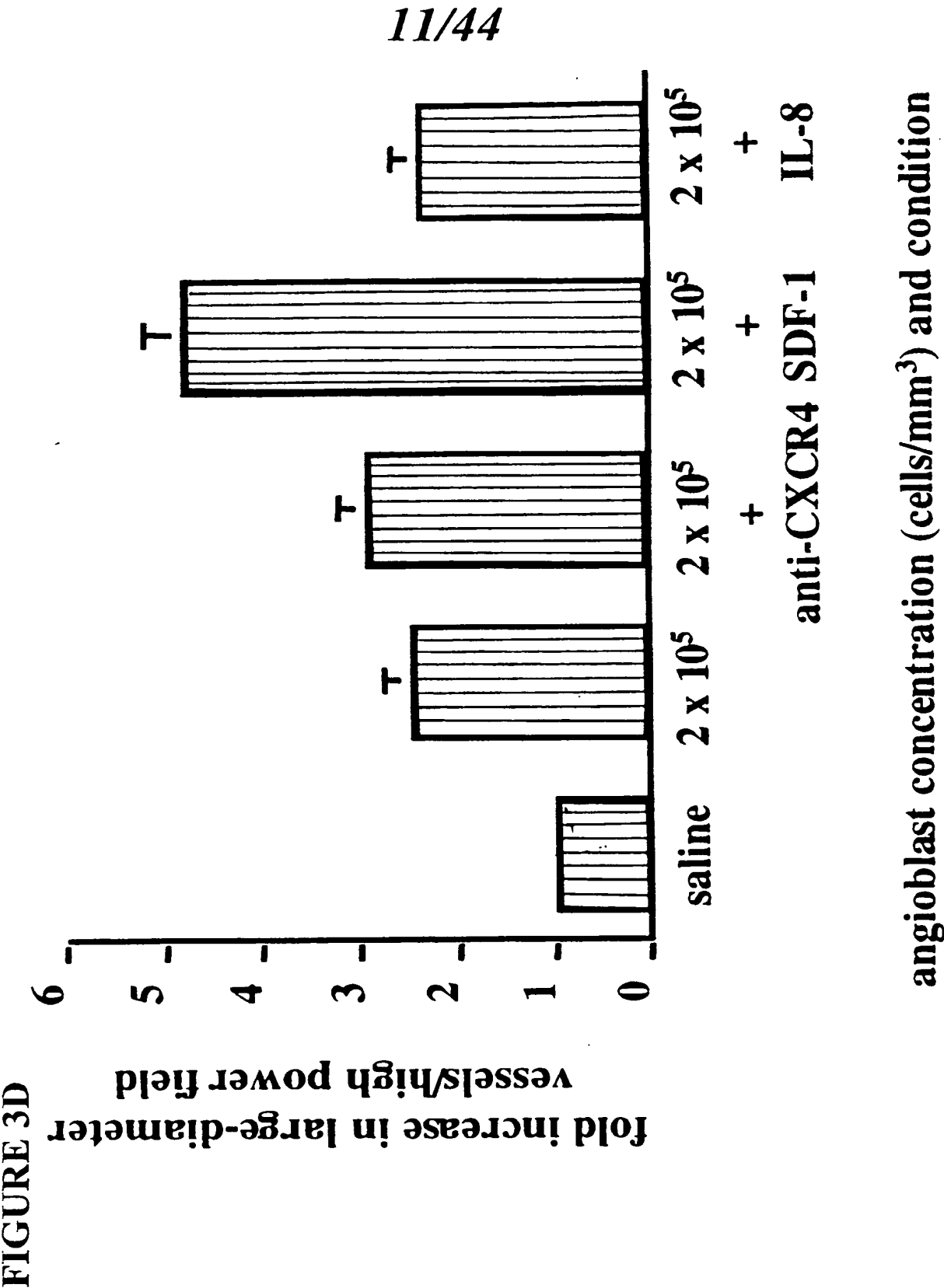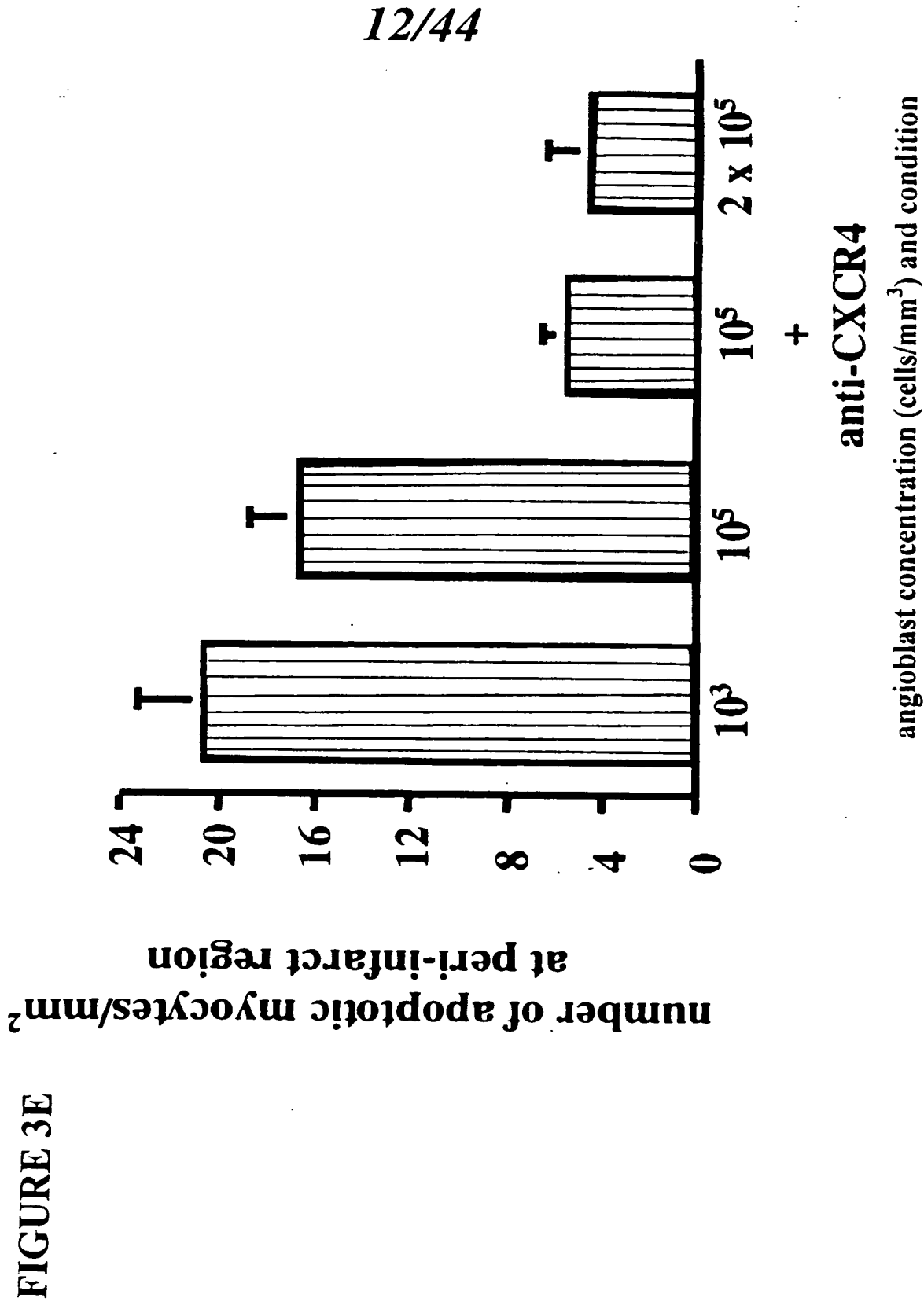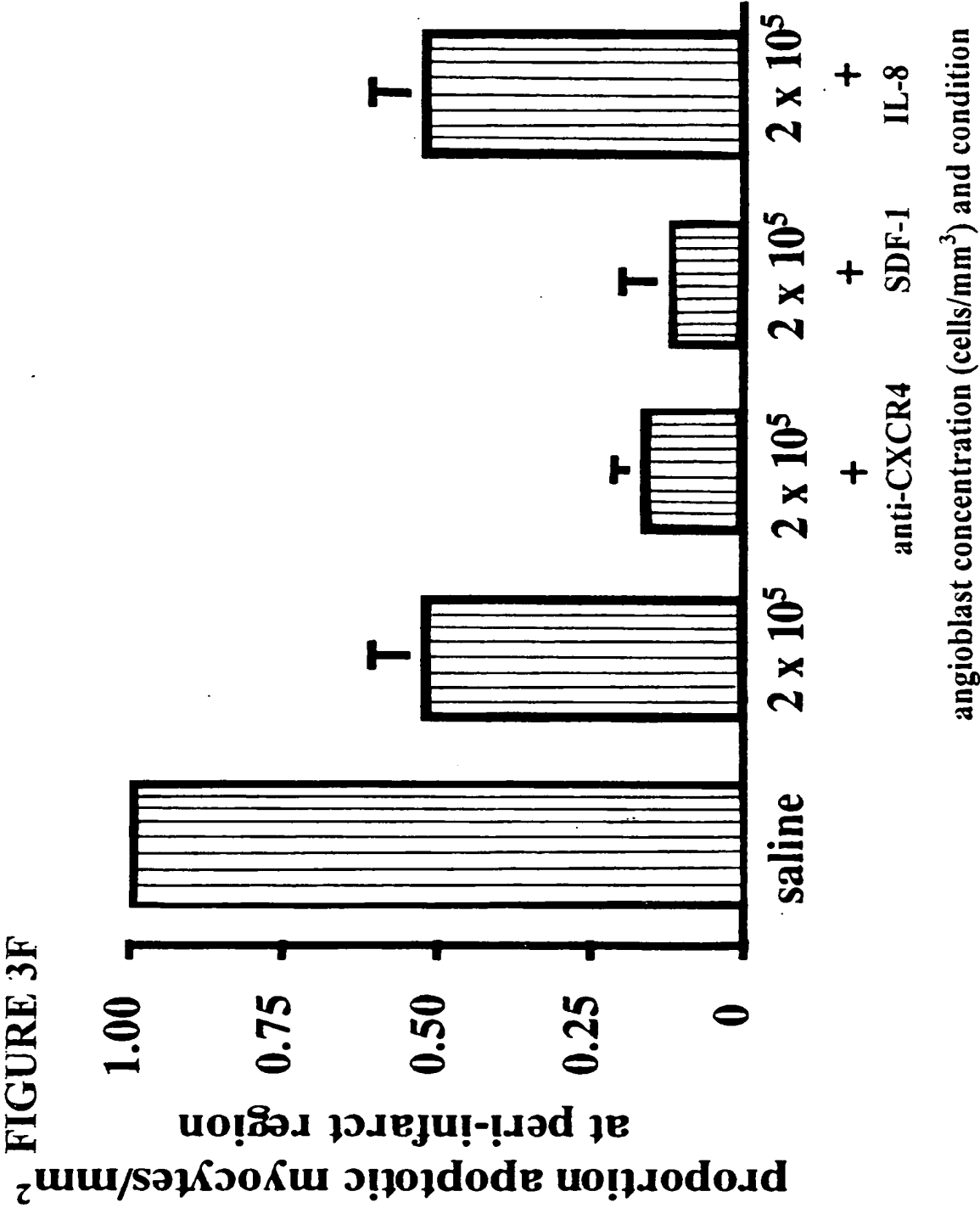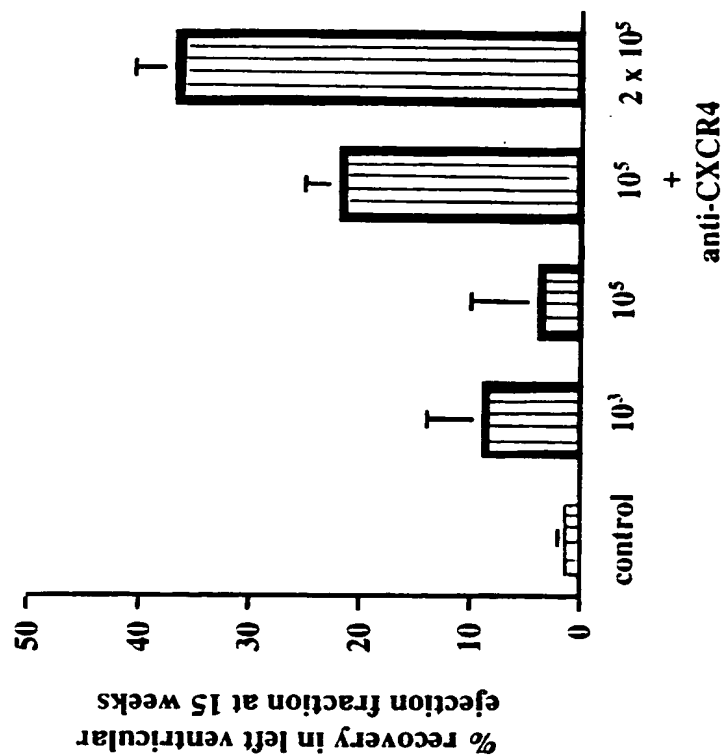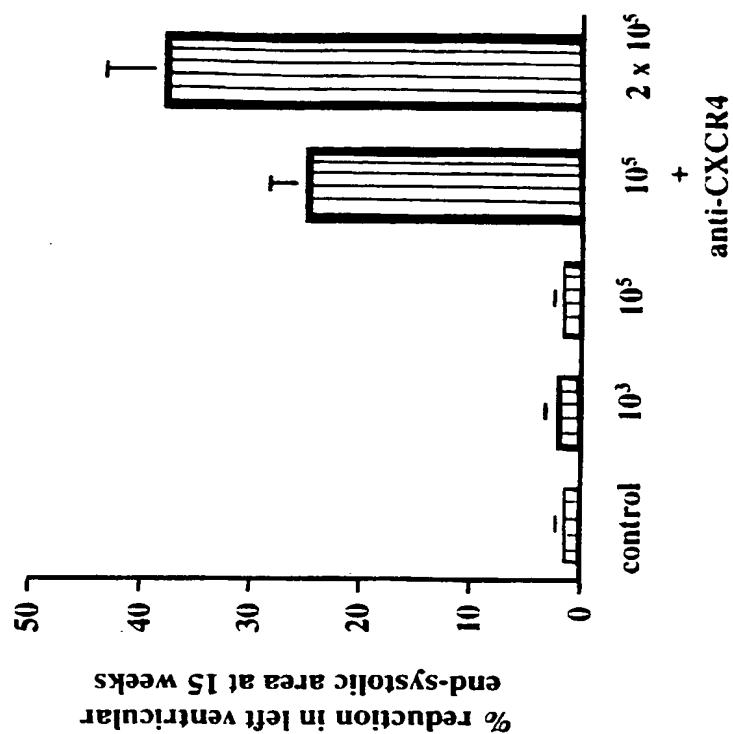
FIGURE 5

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FIGURE 6

```
   1 aaactaaccc ctcttttct ccaaaggagt gcttgtggag atcggatctt ttctccagca
  61 attgggggaa agaaggcttt ttctctgact tcgcttagtg taaccagcgg cgtatatttt
 121 ttaggcgcct tttcgaaaac ctagtagtta atattcattt gtttaaatct tattttattt
 181 ttaagctcaa actgcttaag aataccttaa ttccttaaag tgaaataatt ttttgcaaag
 241 gggtttcctc gatttggagc ttttttttc ttccaccgtc atttctaact cttaaaacca
 301 actcagttcc atcatggtga tgttcaagaa gatcaagtct tttgaggtgg tctttaacga
 361 ccctgaaaag gtgtacggca gtggcgagaa ggtggctggc cgggtgatag tggaggtgtg
 421 tgaagttact cgtgtcaaag ccgttaggat cctggcttgc ggagtggcta aagtgctttg
 481 gatgcaggga tcccagcagt gcaaacagac ttcggagtac ctgcgctatg aagacacgct
 541 tcttctggaa gaccagccaa caggtgagaa tgagatggtg atcatgagac ctggaaacaa
 601 atatgagtac aagttcggct ttgagcttcc tcaggggcct ctgggaacat ccttcaaagg
 661 aaaatatggg tgtgtagact actgggtgaa ggcttttctt gaccgcccga gccagccaac
 721 tcaagagaca aagaaaaact ttgaagtagt ggatctggtg gatgtcaata cccctgattt
 781 aatggcacct gtgtctgcta aaaaagaaaa gaaagtttcc tgcatgttca ttcctgatgg
 841 gcgggtgtct gtctctgctc gaattgacag aaaaggattc tgtgaaggtg atgagatttc
 901 catccatgct gactttgaga atacatgttc ccgaattgtg gtccccaaag ctgccattgt
 961 ggcccgccac acttaccttg ccaatggcca gaccaaggtg ctgactcaga agttgtcatc
1021 agtcagaggc aatcatatta tctcagggac atgcgcatca tggcgtggca agagccttcg
1081 ggttcagaag atcaggcctt ctatcctggg ctgcaacatc cttcgagttg aatattcctt
1141 actgatctat gttagcgttc ctggatccaa gaaggtcatc cttgacctgc ccctggtaat
1201 tggcagcaga tcaggtctaa gcagcagaac atccagcatg gccagccgaa ccagctctga
1261 gatgagttgg gtagatctga acatccctga taccccagaa gctcctccct gctatatgga
1321 tgtcattcct gaagatcacc gattggagag cccaaccact cctctgctag atgacatgga
1381 tggctctcaa gacagcccta tctttatgta tgcccctgag ttcaagttca tgccaccacc
1441 gacttatact gaggtggatc cctgcatcct caacaacaat gtgcagtgag catgtggaag
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1681 agcgcccat ttgtgataca ggggtgtttg ttggatgggt ttaaaaataa ctagaaaaac
1741 tcaggcccat ccattttctc agatctcctt gaaaattgag gccttttcga tagtttcggg
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1861 ttgtgatagg aactttggac cttgaactta cgtatcatgt ggagaagagc caatttaaca
1921 aactaggaag atgaaaaggg aaattgtggc caaaactttg ggaaaaggag gttcttaaaa
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2161 aaggtcttaa aagccatttt tggagcctat tgcactgtgt tctcctactg caaatatttt
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2281 cttagcactt taattcctgt caaattttt gttctcccct tctgccatct taaatgtaag
2341 ctgaaactgg tctactgtgt ctctagggtt aagccaaaag acaaaaaaaa ttttactact
2401 tttgagattg ccccaatgta cagaattata taattctaac gcttaaatca tgtgaaaggg
2461 ttgctgctgt cagccttgcc cactgtgact tcaaacccaa ggaggaactc ttgatcaaga
2521 tgcccaaccc tgtgatcaga acctccaaat actgccatga gaaactagag ggcaggtctt
2581 cataaaagcc ctttgaaccc ccttcctgcc ctgtgttagg agatagggat attggcccct
2641 cactgcagct gccagcactt ggtcagtcac tctcagccat agcactttgt tcactgtcct
2701 gtgtcagagc actgagctcc acccttttct gagagttatt acagccagaa agtgtgggct
2761 gaagatggtt ggtttcatgt
```

FIGURE 7

```
314          ATggtgA Tgttcaagaa gATcaagtct tttgaggtgg tctttaacga
361 ccctgaaaag gtgtacggca gtggcgagaa ggtggctggc cgggtgATag tggaggtgtg
421 tgaagttact cgtgtcaaag ccgttaggAT cctggcttgc ggagtggcta aagtgctttg
481 gATgcaggga Tccagcagt gcaaacagac ttcggagtac ctgcgctATg aagacacgct
541 tcttctggaa gaccagccaa caggtgagaA TgagATggtg ATcATgagac ctggaaacaa
601 ATATgagtac aagttcggct ttgagcttcc tcaggggcct ctgggaacAT cccttcaaagg
661 aaaATATggg tgtgtagact actgggtgaa ggcttttctt gaccgcccga gccagccaac
721 tcaagagaca aagaaaaact ttgaagtagt ggATctggtg gATgtcaATa cccctgATtt
781 aATggcacct gtgtctgcta aaaaagaaaa gaaagtttcc tgcATgttcA TtcctgATgg
841 gcgggtgtct gtctctgctc gaATtgacag aaaaggATtc tgtgaaggtg ATgagATttc
901 cATccATgct gactttgaga ATacATgttc ccgaATtgtg gtccccaaag ctgccATtgt
961 ggcccgccac acttaccttg ccaATggcca gaccaaggtg ctgactcaga agttgtcATc
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1081 ggttcagaag ATcaggcctt ctATcctggg ctgcaacATc cttcgagttg aATATtcctt
1141 actgATctAT gttagcgttc ctgATccaa gaaggtcATc cttgacctgc ccctggtaAT
1201 tggcagcagA Tcaggtctaa gcagcagaac ATccagcATg gccagccgaa ccagctctga
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1321 TgtcATtcct gaagATcacc gATtggagag cccaaccact cctctgctag ATgacATggA
1381 Tggctctcaa gacagccctA TctttATgtA Tgcccctgag ttcaagttcA Tgccaccacc
1441 gacttATact gaggtggATc cctgcATcct caacaacaAT gtgcagtga
```

FIGURE 8

```
314      atggtga tgttcaagaa gatcaagtct tttgaggtgg tctttaacga
361 ccctgaaaag gtgtacgGCa gtgGCggagaa ggtgGCtgGC cgggtgatag tggaggtgtg
421 tgaagttact cgtgtcaaaG Ccgttaggat cctgGCttGC ggagtgGCta aagtGCtttg
481 gatGCaggga tcccaGCagt GCaaacagac ttcggagtac ctGCGCtatg aagacacGCt
541 tcttctggaa gaccaGCcaa caggtgagaa tgagatggtg atcatgagac ctggaaacaa
601 atatgagtac aagttcgGCt ttgaGCttcc tcagggGCct ctggaacat cttcaaagg
661 aaaatatggg tgtgtagact actgggtgaa gGCtttcctt gaccGCccga GCcaGCcaac
721 tcaagagaca aagaaaaact ttgaagtagt ggatctggtg gatgtcaata ccctgatt
781 aatgGCcacct gtgtctGCta aaaaagaaaa gaaagtttcc tGCatgttca ttcctgatgg
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901 catccatGCt gactttgaga atacatgttc ccgaattgtg gtcccaaaG CtGCcattgt
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1381 tgGCtctcaa gacaGCccta tctttatgta tGCccctgag ttcaagttca tGCCaccacc
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```

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FIGURE 9

```
                   atgGTga   tGTtcaagaa  gatcaaGTct  tttgagGTgG  Tctttaacga
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541  tcttctggaa  gaccagccaa  cagGTgagaa  tgagatgGTg  atcatgagac  ctggaaacaa
601  atatgaGTac  aaGTtcggct  ttgagcttcc  tcaggggcct  ctgggaacat  ccttcaaagg
661  aaaatatggG  TGTGTagact  actggGTgaa  ggcttttctt  gaccgcccga  gccagccaac
721  tcaagagaca  aagaaaaact  ttgaaGTaGT  ggatctgGTg  gatGTcaata  cccctgattt
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1081 gGTtcagaag  atcaggcctt  ctatcctggg  ctgcaacatc  cttcgaGTtg  aatattcctt
1141 actgatctat  GTtagcGTtc  ctggatccaa  gaagGTcatc  cttgacctgc  ccctgGTaat
1201 tggcagcaga  tcagGTctaa  gcagcagaac  atccagcatg  gccagccgaa  ccagctctga
1261 gatgaGTtgg  GTagatctga  acatccctga  tacccagaa   gctcctccct  gctatatgga
1321 tGTtcattcct  gaagatcacc  gattggagag  cccaaccact  cctctgctag  atgacatgga
1381 tggctctcaa  gacagcccta  tctttatGTa  tgcccctgaG  TtcaaGTtca  tgccaccacc
1441 gacttatact  gagGTtggatc  cctgcatcct  caacaacaat  GtgcaGTga
```

FIGURE 10

```
314        atggtga tgttcaagaa gatcaagtct tttgaggtgg tctttaACgA
361 Ccctgaaaag gtgtACggca gtggcgagaa ggtggctggc cgggtgatag tggaggtgtg
421 tgaagttACt cgtgtcaaag ccgttaggat cctggcttgc ggagtggcta aagtgctttg
481 gatgcaggga tcccagcagt gcaaACagAC ttcggagtAC ctgcgctatg aagACACgct
541 tcttctggaa gACcagccaA Caggtgagaa tgagatggtg atcatgagAC ctggaaACaa
601 atatgagtAC aagttcggct ttgagcttcc tcaggggcct cctttcaaagg cctttcaaagg
661 aaaatatggg tgtgtagACt ACtgggtgaa ggctttctt gACcgcccga gccagccaAC
721 tcaagagACa aagaaaaAct ttgaagtagt ggatcctggtg gatgtcaatA Ccctgattt
781 aatggcACct gtgtctgcta aaaagaaaaa gaaagtttcc tgcatgttca ttcctgatgg
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FIGURE 11

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 541 TcTTctggaa gaccagccaa caggtgagaa tgagatggtg aTcatgagac ctggaaacaa
 601 aTatgagTac aagTTcggcT TtgagcTTcc TcaggggccT ctgggaacaT ccTTcaaagg
 661 aaaaTatggg tgtgTagacT actgggtgaa ggcTTTTcTt gaccgcccga gccagccaac
 721 Tcaagagaca aagaaaaacT TtgaagTagt ggaTctggtg gatgTcaaTa CCCctgaTTT
 781 aatggcacct gtgTctgcTa aaaaagaaaa gaaagTTTcc tgcatgTTca TTcctgatgg
 841 gcgggtgTct gTcTctgcTc gaaTtgacag aaaggaTTTc tgtgaaggtg atgagaTTTc
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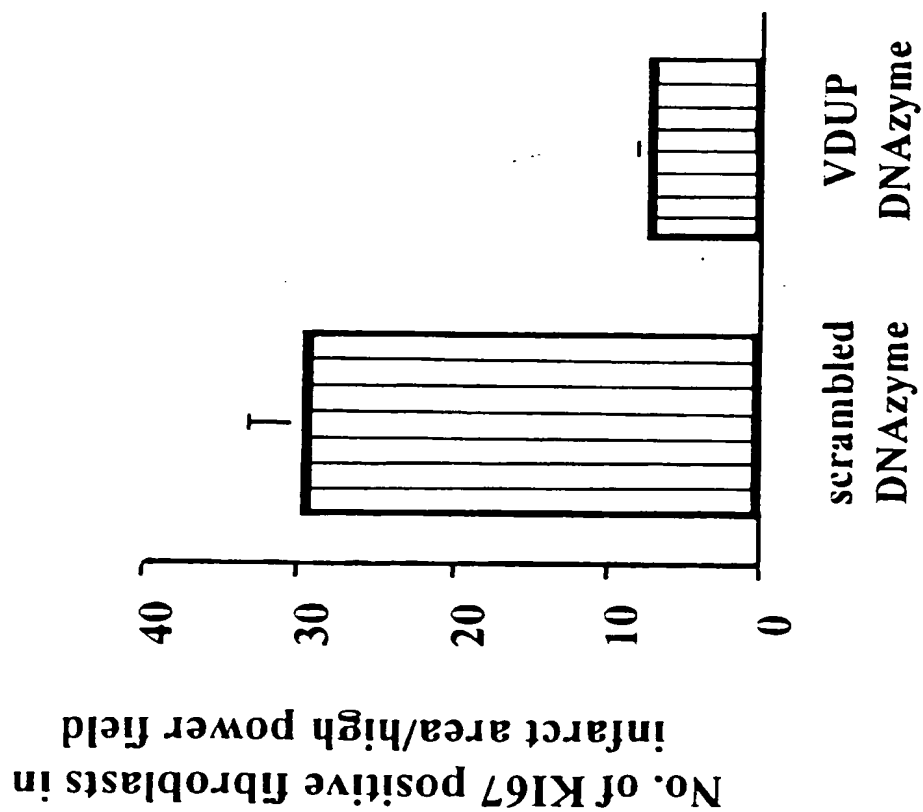
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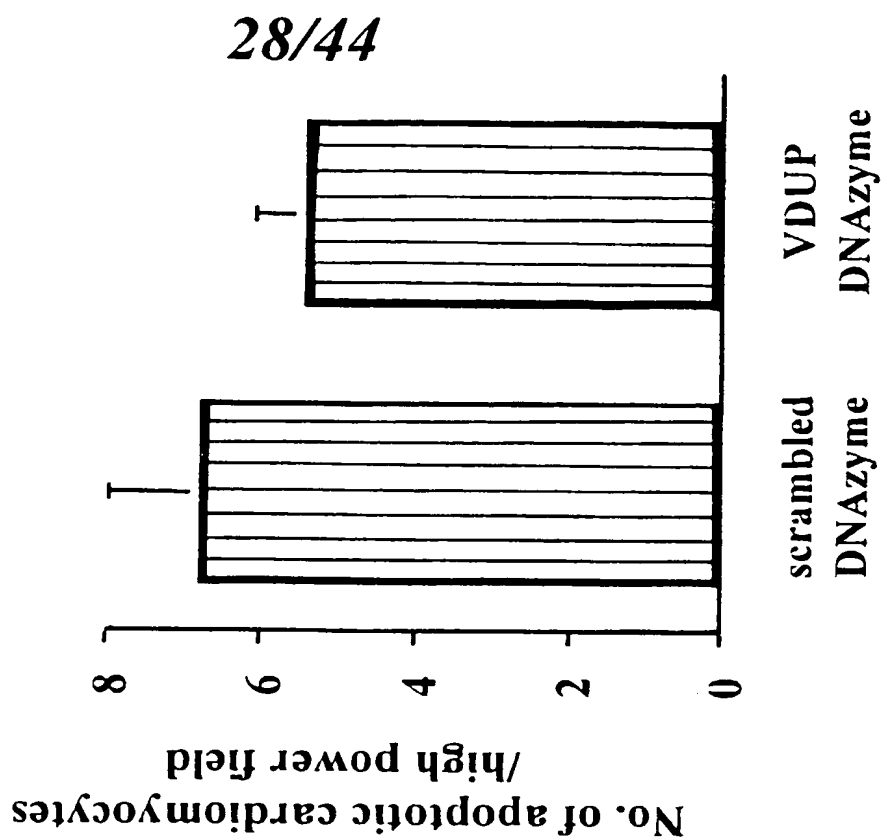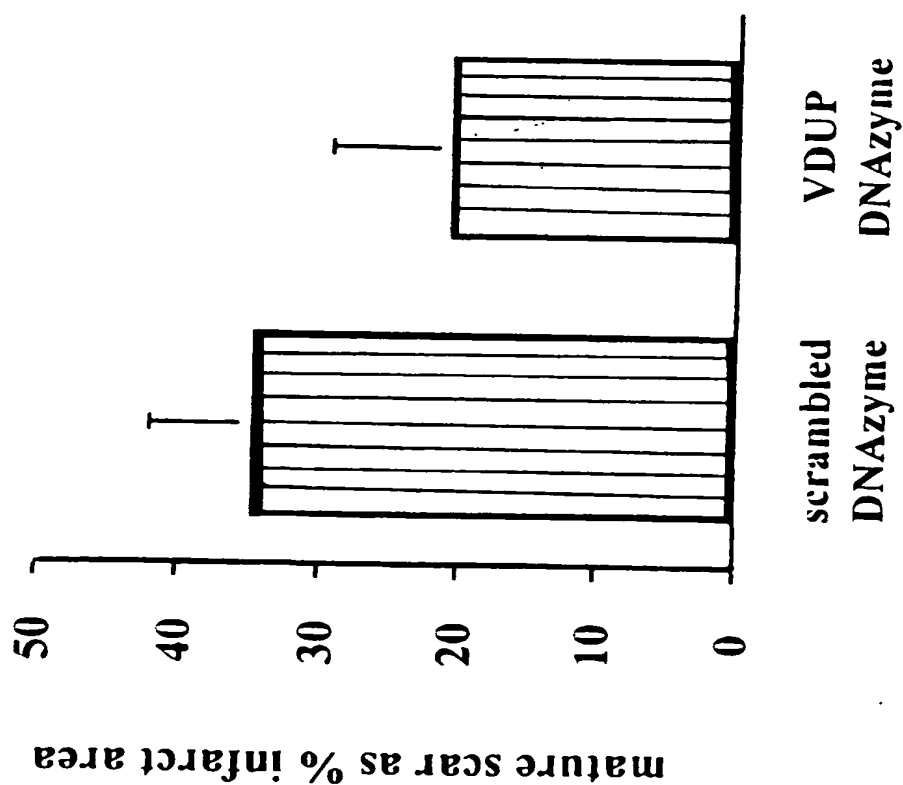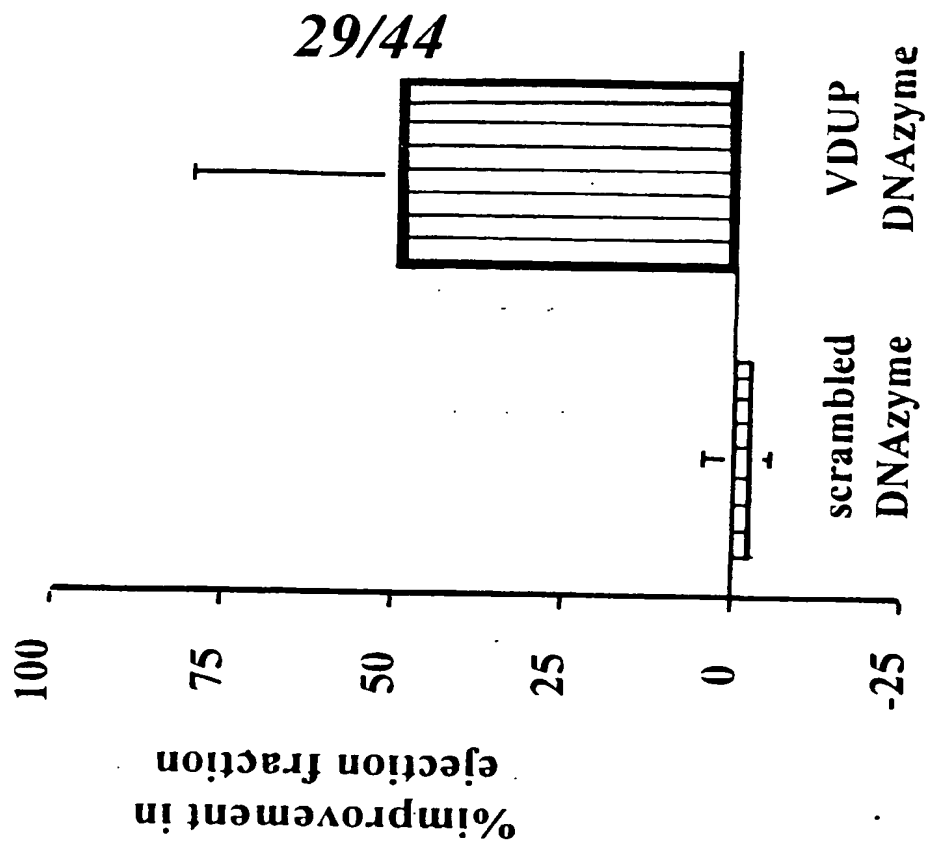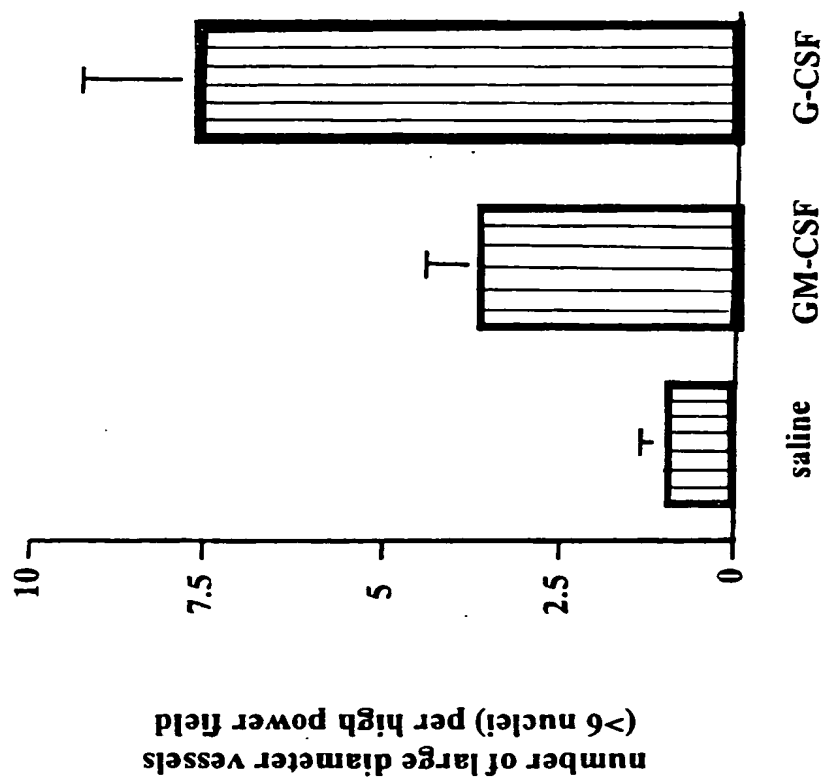
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FIGURE 13B

FIGURE 13A

FIGURE 14B

FIGURE 14A

FIGURE 15B

FIGURE 15A

FIGURE 16B

% change in ejection fractionbetween 2 days and
2 weeks post myocardial infarction

FIGURE 16A

fold increase in rat cardiomyocytes
entering cell cycle relative to saline controls

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FIGURE 17B

FIGURE 17A

FIGURE 18

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FIGURE 19B

% reduction in apoptotic myocytes at peri-infarct region relative to saline

FIGURE 19A

number of large diameter vessels (>6 nuclei) per high power field

FIGURE 20B

(b)

% change in ejection fraction between 2 days and
2 weeks post myocardial infarction

FIGURE 20A

(a)

fold increase in rat cardiomyocytes
entering cell cycle relative to saline controls

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FIGURE 21B

proportion of apoptotic myocytes
at peri-infarct region

saline   angioblasts

1.0   .75   .50   .25   0

FIGURE 21A

fold increase in vessels/high power
field at peri-infarct region

4   3   2   1   0

saline   angioblasts

FIGURE 22A

Early Proliferation/ Regeneration Of Immature Cardiomyocyte.
Lineage Progenitors Accompanying Neovascularization

(confocal microscopy: red nucleus, yellow Ki67, blue cytoplasm alpha-sarcomeric actin)

(a)
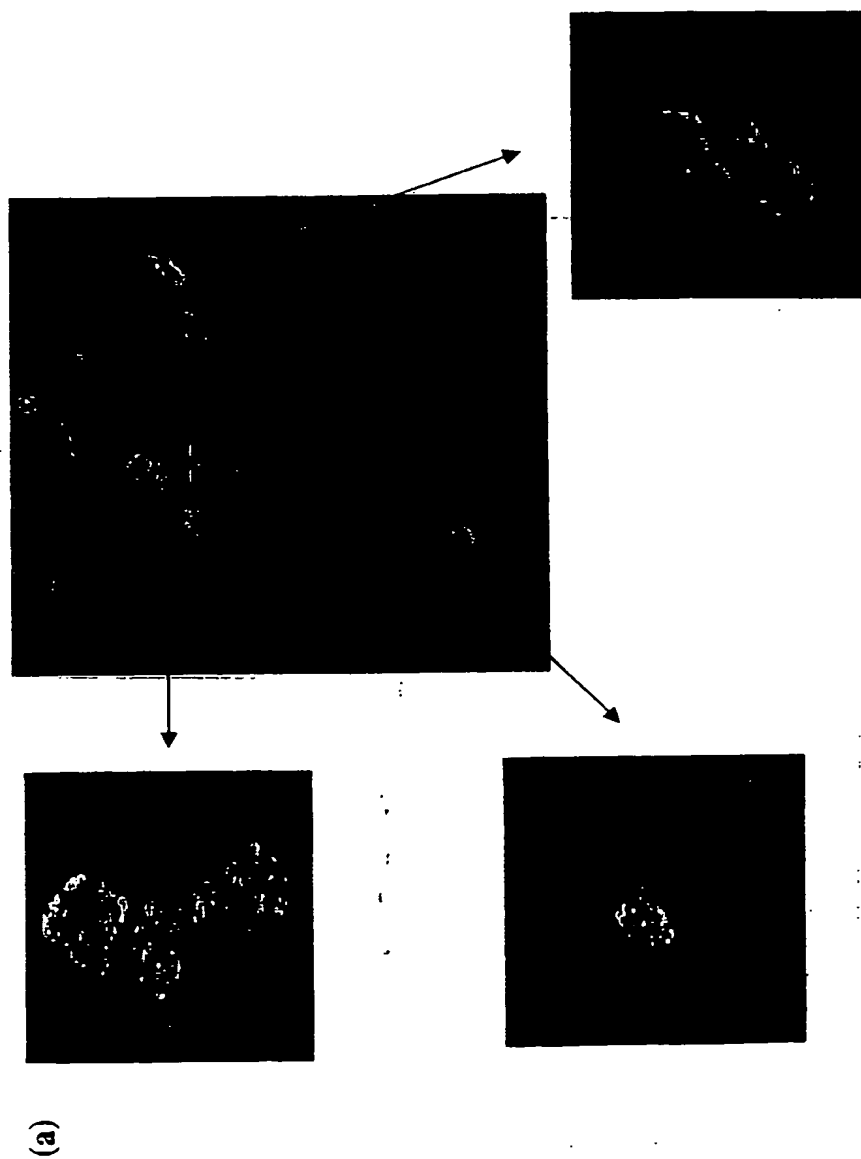
FIGURE 22B

**Later Differentiation And Regeneration Of Mature Cardiomyocytes
Accompanying Neovascularization**

(immunohistochemistry: blue nucleus rat Ki67, brown cytoplasm troponin I)
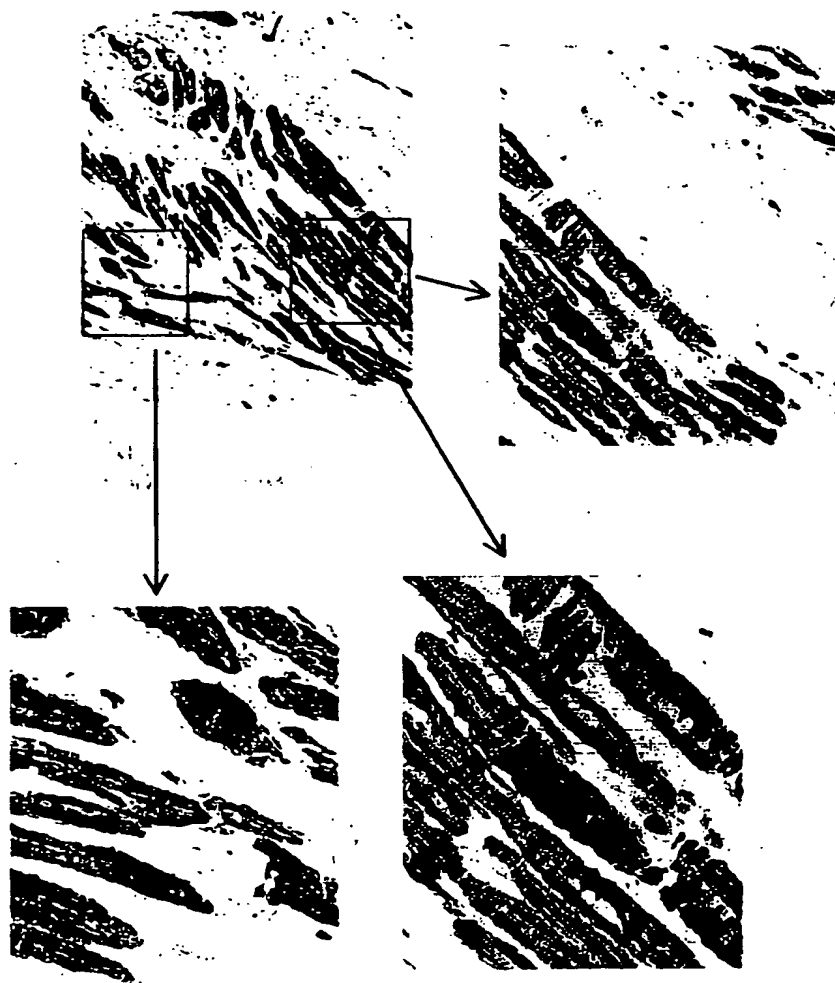
FIGURE 22C

(c)
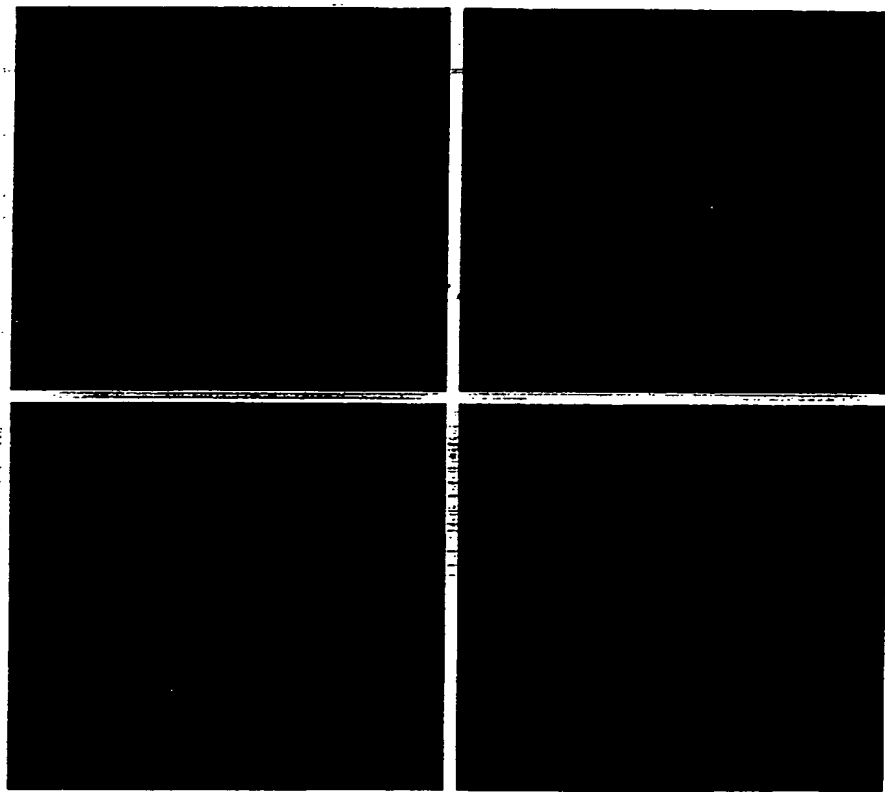
Confocal microscopy showing nuclear cycling (blue nucleus, green rat Ki67) of troponin I-positive mature cardiomyocyte (red cytoplasm)
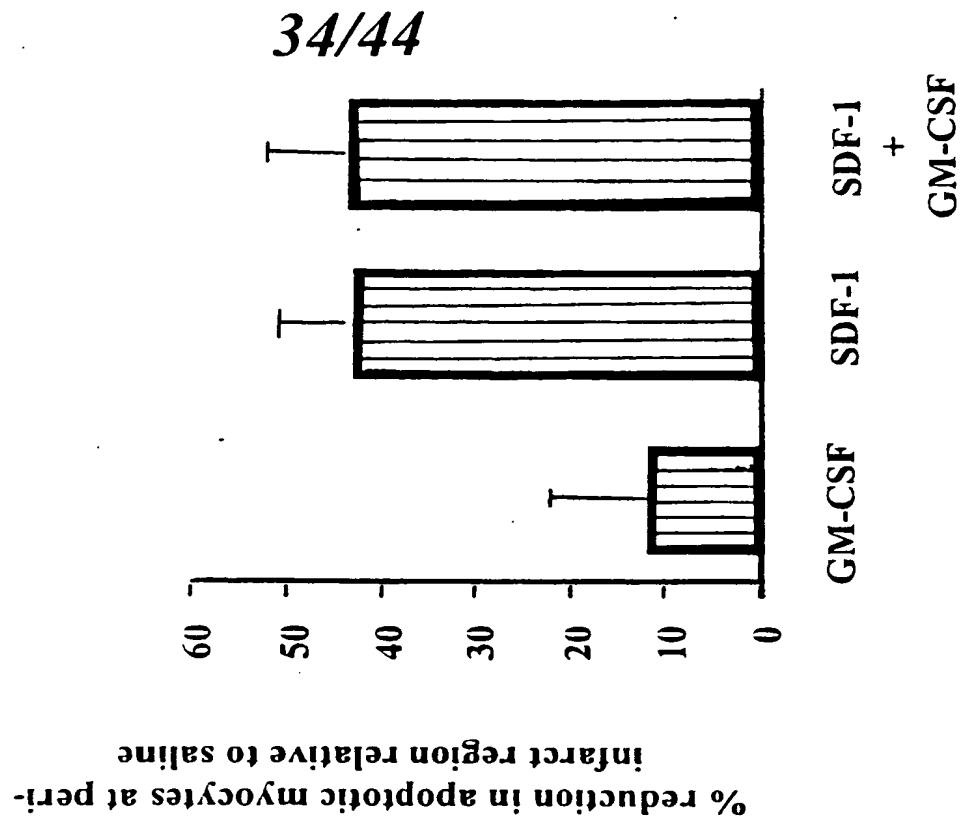
FIGURE 23

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FIGURE 24A (a)

FIGURE 24B (b)

FIGURE 25A

FIGURE 25B

FIGURE 25C
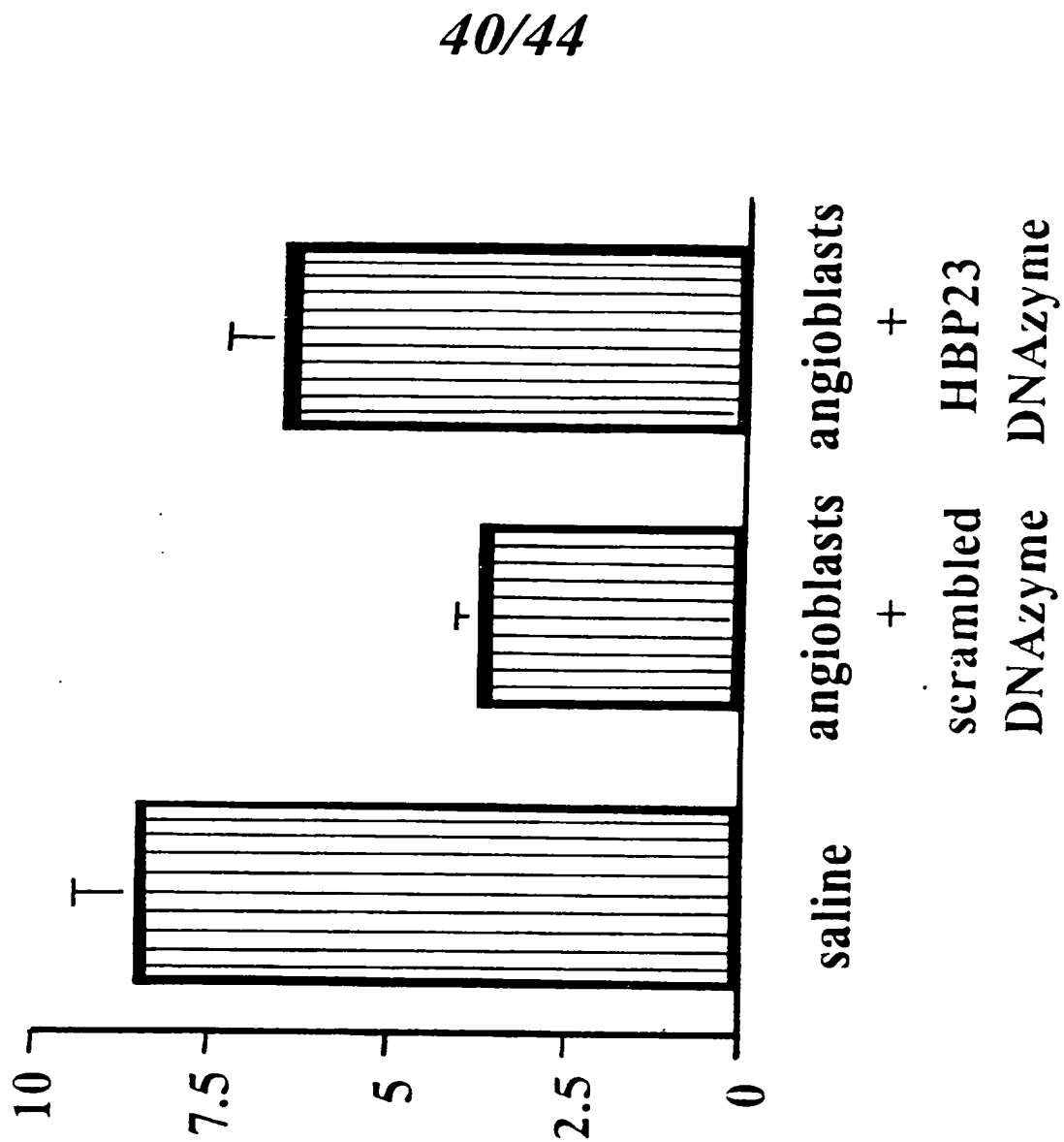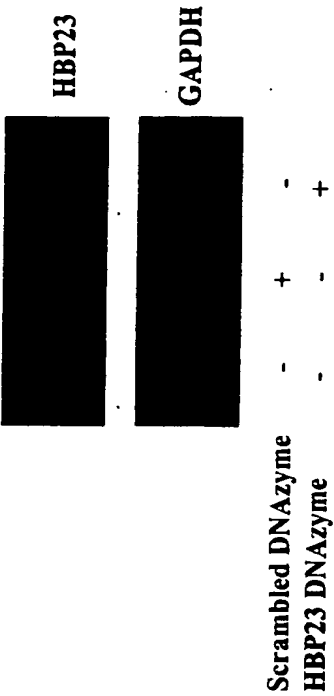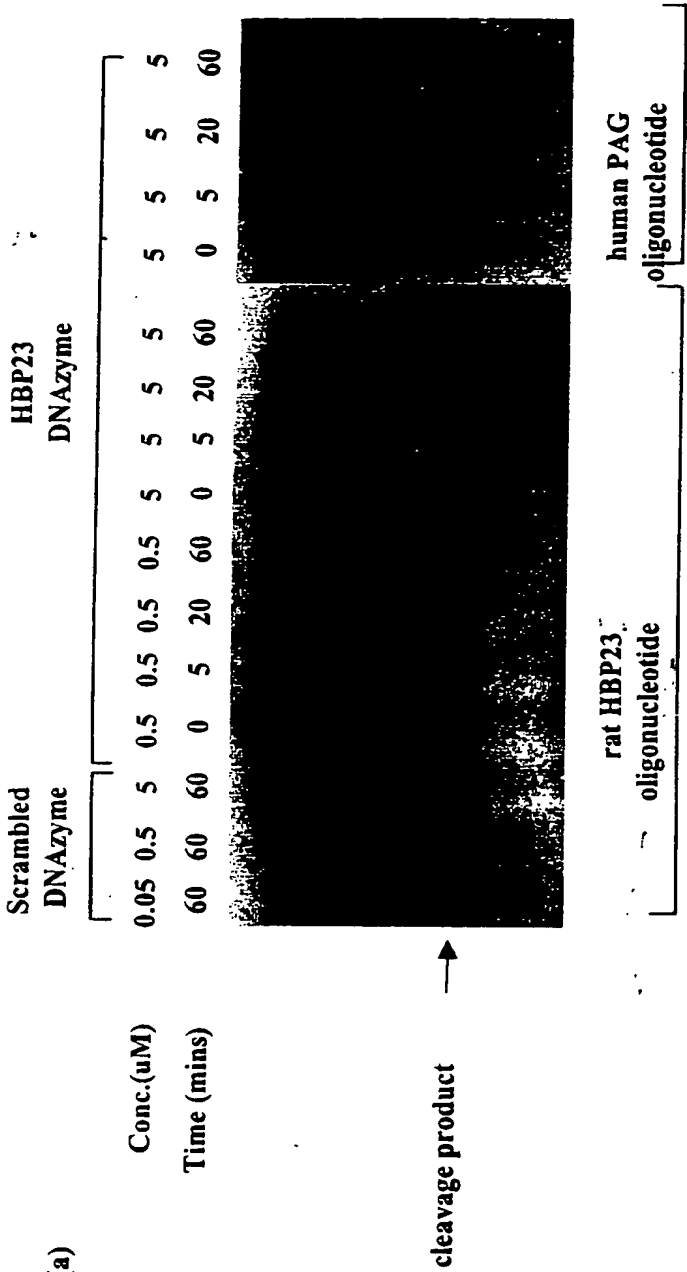
SEQUENCE LISTING

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<120> REGENERATION OF ENDOGENOUS MYOCARDIAL TISSUE BY INDUCTION OF NEOVASCULARIZA
TION

<130> 0575/66602-A-PCT

<140> NOT YET KNOWN
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ttaagctcaa actgcttaag aataccttaa ttccttaaag tgaaataatt ttttgcaaag   240

gggtttcctc gatttggagc tttttttttc ttccaccgtc atttctaact cttaaaacca   300

actcagttcc atc atg gtg atg ttc aag aag atc aag tct ttt gag gtg   349
            Met Val Met Phe Lys Lys Ile Lys Ser Phe Glu Val
            1   5   10

gtc ttt aac gac cct gaa aag gtg tac ggc agt ggc gag aag gtg gct   397
Val Phe Asn Asp Pro Glu Lys Val Tyr Gly Ser Gly Glu Lys Val Ala
        15   20   25

ggc cgg gtg ata gtg gag gtg tgt gaa gtt act cgt gtc aaa gcc gtt   445
Gly Arg Val Ile Val Glu Val Cys Glu Val Thr Arg Val Lys Ala Val
    30   35   40

agg atc ctg gct tgc gga gtg gct aaa gtg ctt tgg atg cag gga tcc   493
Arg Ile Leu Ala Cys Gly Val Ala Lys Val Leu Trp Met Gln Gly Ser
45   50   55   60

cag cag tgc aaa cag act tcg gag tac ctg cgc tat gaa gac acg ctt   541
Gln Gln Cys Lys Gln Thr Ser Glu Tyr Leu Arg Tyr Glu Asp Thr Leu
                65   70   75

ctt ctg gaa gac cag cca aca ggt gag aat gag atg gtg atc atg aga   589
Leu Leu Glu Asp Gln Pro Thr Gly Glu Asn Glu Met Val Ile Met Arg
                80   85   90

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Pro Gly Asn Lys Tyr Glu Tyr Lys Phe Gly Phe Glu Leu Pro Gln Gly
        95                      100                 105

cct ctg gga aca tcc ttc aaa gga aaa tat ggg tgt gta gac tac tgg      685
Pro Leu Gly Thr Ser Phe Lys Gly Lys Tyr Gly Cys Val Asp Tyr Trp
    110                     115                 120

gtg aag gct ttt ctt gac cgc ccg agc cag cca act caa gag aca aag      733
Val Lys Ala Phe Leu Asp Arg Pro Ser Gln Pro Thr Gln Glu Thr Lys
125                     130                 135                 140

aaa aac ttt gaa gta gtg gat ctg gtg gat gtc aat acc cct gat tta      781
Lys Asn Phe Glu Val Val Asp Leu Val Asp Val Asn Thr Pro Asp Leu
                    145                 150                 155

atg gca cct gtg tct gct aaa aaa gaa aag aaa gtt tcc tgc atg ttc      829
Met Ala Pro Val Ser Ala Lys Lys Glu Lys Lys Val Ser Cys Met Phe
                160                 165                 170

att cct gat ggg cgg gtg tct gtc tct gct cga att gac aga aaa gga      877
Ile Pro Asp Gly Arg Val Ser Val Ser Ala Arg Ile Asp Arg Lys Gly
            175                 180                 185

ttc tgt gaa ggt gat gag att tcc atc cat gct gac ttt gag aat aca      925
Phe Cys Glu Gly Asp Glu Ile Ser Ile His Ala Asp Phe Glu Asn Thr
        190                 195                 200

tgt tcc cga att gtg gtc ccc aaa gct gcc att gtg gcc cgc cac act      973
Cys Ser Arg Ile Val Val Pro Lys Ala Ala Ile Val Ala Arg His Thr
205                 210                 215                 220

tac ctt gcc aat ggc cag acc aag gtg ctg act cag aag ttg tca tca    1021
Tyr Leu Ala Asn Gly Gln Thr Lys Val Leu Thr Gln Lys Leu Ser Ser
                225                 230                 235

gtc aga ggc aat cat att atc tca ggg aca tgc gca tca tgg cgt ggc    1069
Val Arg Gly Asn His Ile Ile Ser Gly Thr Cys Ala Ser Trp Arg Gly
            240                 245                 250

aag agc ctt cgg gtt cag aag atc agg cct tct atc ctg ggc tgc aac    1117
Lys Ser Leu Arg Val Gln Lys Ile Arg Pro Ser Ile Leu Gly Cys Asn
        255                 260                 265

atc ctt cga gtt gaa tat tcc tta ctg atc tat gtt agc gtt cct gga    1165
Ile Leu Arg Val Glu Tyr Ser Leu Leu Ile Tyr Val Ser Val Pro Gly
    270                 275                 280

tcc aag aag gtc atc ctt gac ctg ccc ctg gta att ggc agc aga tca    1213
Ser Lys Lys Val Ile Leu Asp Leu Pro Leu Val Ile Gly Ser Arg Ser
285                 290                 295                 300

ggt cta agc agc aga aca tcc agc atg gcc agc cga acc agc tct gag    1261
Gly Leu Ser Ser Arg Thr Ser Ser Met Ala Ser Arg Thr Ser Ser Glu
                305                 310                 315

atg agt tgg gta gat ctg aac atc cct gat acc cca gaa gct cct ccc    1309
Met Ser Trp Val Asp Leu Asn Ile Pro Asp Thr Pro Glu Ala Pro Pro
            320                 325                 330
```

Page 2

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Cys Tyr Met Asp Val Ile Pro Glu Asp His Arg Leu Glu Ser Pro Thr
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Thr Pro Leu Leu Asp Asp Met Asp Gly Ser Gln Asp Ser Pro Ile Phe
        350                 355                 360

atg tat gcc cct gag ttc aag ttc atg cca cca ccg act tat act gag      1453
Met Tyr Ala Pro Glu Phe Lys Phe Met Pro Pro Pro Thr Tyr Thr Glu
365                 370                 375                 380

gtg gat ccc tgc atc ctc aac aac aat gtg cag tga gcatgtggaa          1499
Val Asp Pro Cys Ile Leu Asn Asn Asn Val Gln
                385                 390
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Cys Gly Val Ala Lys Val Leu Trp Met Gln Gly Ser Gln Gln Cys Lys
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Gln Thr Ser Glu Tyr Leu Arg Tyr Glu Asp Thr Leu Leu Leu Glu Asp
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Gln Pro Thr Gly Glu Asn Glu Met Val Ile Met Arg Pro Gly Asn Lys
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Tyr Glu Tyr Lys Phe Gly Phe Glu Leu Pro Gln Gly Pro Leu Gly Thr
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Ser Phe Lys Gly Lys Tyr Gly Cys Val Asp Tyr Trp Val Lys Ala Phe
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Leu Asp Arg Pro Ser Gln Pro Thr Gln Glu Thr Lys Lys Asn Phe Glu
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Val Val Asp Leu Val Asp Val Asn Thr Pro Asp Leu Met Ala Pro Val
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Ser Ala Lys Lys Glu Lys Lys Val Ser Cys Met Phe Ile Pro Asp Gly
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Arg Val Ser Val Ser Ala Arg Ile Asp Arg Lys Gly Phe Cys Glu Gly
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Asp Glu Ile Ser Ile His Ala Asp Phe Glu Asn Thr Cys Ser Arg Ile
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Val Val Pro Lys Ala Ala Ile Val Ala Arg His Thr Tyr Leu Ala Asn
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Gly Gln Thr Lys Val Leu Thr Gln Lys Leu Ser Ser Val Arg Gly Asn
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His Ile Ile Ser Gly Thr Cys Ala Ser Trp Arg Gly Lys Ser Leu Arg
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Val Gln Lys Ile Arg Pro Ser Ile Leu Gly Cys Asn Ile Leu Arg Val
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Glu Tyr Ser Leu Leu Ile Tyr Val Ser Val Pro Gly Ser Lys Lys Val
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Ile Leu Asp Leu Pro Leu Val Ile Gly Ser Arg Ser Gly Leu Ser Ser
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Arg Thr Ser Ser Met Ala Ser Arg Thr Ser Ser Glu Met Ser Trp Val
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Asp Leu Asn Ile Pro Asp Thr Pro Glu Ala Pro Pro Cys Tyr Met Asp
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Val Ile Pro Glu Asp His Arg Leu Glu Ser Pro Thr Thr Pro Leu Leu
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Asp Asp Met Asp Gly Ser Gln Asp Ser Pro Ile Phe Met Tyr Ala Pro
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gtcaaagccg ttaggatcct ggcttgcgga gtggctaaag tgctttggat gcagggatcc · 180

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